10-17-2018 11:30 AM

Extraction of Astaxanthin from H. pluvialis Using Deep Eutectic Solvents

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Graduate Program in Chemical and Biochemical Engineering
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Engineering Science

Abstract

Haematococcus pluvialis (H. pluvialis) is one of the main sources of astaxanthin, a high-value carotenoid and a strong antioxidant. Astaxanthin extraction is hindered by the thick cell wall of *H. pluvialis* which is composed of sporopollenin. Mechanical cell disruption methods, high cost and energy-consuming, are required to enhance astaxanthin extraction. This study employed a novel technology using deep eutectic solvents (DESs) to demonstrate the possibility of direct extraction of astaxanthin without a disruption step. It was established that 68% of astaxanthin can be extracted from intact *H. pluvialis* cells using choline chloride-glycerol (ChCl-G) mixture with 12.5% (v/v) water at 70 °C. Using deep eutectic solvents is a promising method for efficient extraction of astaxanthin from *H. pluvialis*, while complying with Green Chemistry Principles.

Keywords

Microalgae, *H. pluvialis*, astaxanthin, deep eutectic solvent, choline chloride based-deep eutectic solvents, extraction, central composite design.

Acknowledgments

First, I would like to thank and acknowledge the guidance provided by my supervisor Dr. Lars Rehmann who this was impossible without his support.

I also thank my labmates in this two years including Erin Johnson, Yi-kai Su, Claudio Silva Lira, Garret Munch, Yujie Zhang, Connie Wen, Priyanka Saxena and Colin Couper who helped and supported me a lot during my research and made working in the lab fun.

Finally, I would also like to thank my family and friends, especially my mom who supported me were there for me when things were hard and encouraged me to keep going.

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Chapter 1

1 Introduction

Microalgae are important and diverse groups of microorganisms which are commonly found in oceans, rivers, freshwater lakes and ponds (Sahoo & Seckbach, 2015). Their size can vary from a few micrometers to a few hundred micrometers (Singh & Saxena, 2015). Producing biofuel from microalgae was one of the first applications that attracted researchers to studying microalgae, but they later found out that microalgae can also be a good source of other products such as high-value organic chemicals for pharmaceuticals and nutraceuticals, pigments and livestock feed (Shah, Liang, Cheng, & Daroch, 2016). *H. pluvialis* is one of more valuable microalgae, as it is the main sources of astaxanthin, which is one of the strongest natural antioxidants and has shown different health benefits (José, Reyes, Mendiola, & Iba, 2014). The emerging area of green chemistry encourages both industry and academia to design and use chemicals and processes that would decrease the use of hazardous materials because of environmental and health concerns(Mbous et al., 2017). As part of the shift toward green chemistry, extensive research on the development and application of a group of organic salts with melting points below 100°C is currently being conducted so that researchers and industry professionals can replace volatile and hazardous solvents that are currently used (Pena-Pereira & Namieśnik, 2014). These solvents possess distinct physicochemical properties: they are thermally stable, non-volatile, non-flammable and their miscibility and polarity are tunable to a great extent.

A brief introduction to *H. pluvialis*, astaxanthin and astaxanthin extraction technologies are presented in this chapter.

1.1 *Haematococcus pluvialis*

1.1.1.1 Life cycle of *H. pluvialis*

H. pluvialis is a green, freshwater microalga that accumulates large amounts of astaxanthin, up to 2-4% (w/w) on a dry weight basis and has gained commercial importance as one of the most important natural sources of astaxanthin (Ratledge, 1982). This strain of microalgae has a rather complex life cycle: it has three distinct stages, including motile flagellated zooids which are called macrozooids (zoospores), palmella and hematocysts (aplanospores) (Shah et al., 2016). The first two phases are called "green or vegetative stage," and the last phase is called "red or encysted stage." Macrozooid, motile and biflagellate, cells are between 8 and 20 μm long and grow quickly under favorable conditions, but when the conditions are unfavorable for growth, they lose their flagella, expand their size and transform into non-motile cells (Hagen, Siegmund, & Braune, 2002). If extreme conditions continue and the stress on the microalgae remains high, the green vegetative cells start to build a thick cell wall, growth stops and the cells transform into aplanospores and accumulate astaxanthin (Boussiba & Vonshak, 1991).

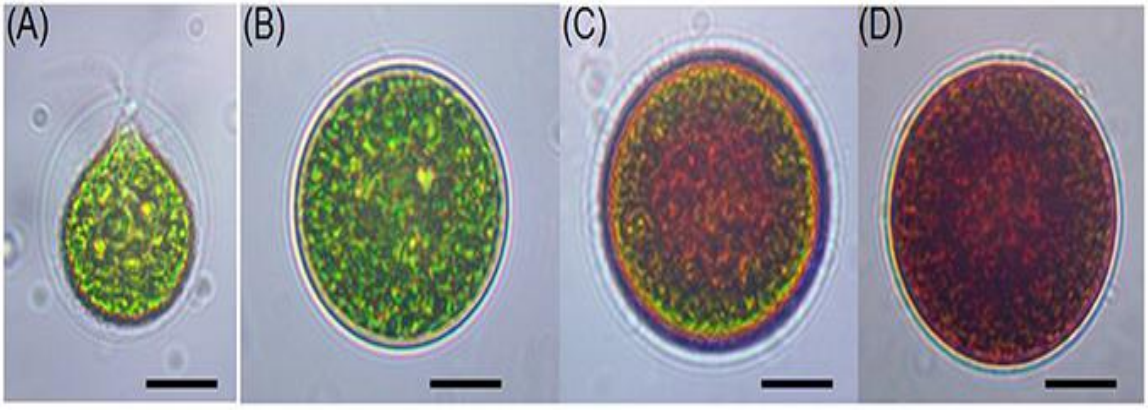

Figure 1: *H. pluvialis* life cycle under a light microscope. (A) Macrozooid cells; (B) Palmella cells; (C) Transition step to the red stage; (D) Aplanospore cells. Scale bar: 10μm (Shah et al., 2016)**.**

Freshwater algae are unprotected and usually face harsh environmental conditions such as extreme temperature, dryness and nutrient deficiency. In order to survive in severe conditions, microalgae have developed strategies and mechanisms that protect them. In

poor conditions, *Haematococcus* builds a rigid trilaminar cell wall and stops all metabolic activities (Fátima Santos & Mesquita, 1984). Main components of *Haematococcus* cell wall at the encysted stage is 70% carbohydrates, 6% proteins, 3% cellulose and 3% acetolysis-resistant material (Hagen et al., 2002). Astaxanthin accumulated in aplanospore cells deposits in lipid droplets around the nucleus and gives bright pinkish-red color to the cells (Hagen et al., 2002). One of the main problems faced in astaxanthin and other carotenoid extraction from *Haematococcus* is its thick cell wall. Researchers reported that although astaxanthin existed in intact *H. pluvialis,* it was not bioavailable as the fish that were fed the uncracked cells did not absorb astaxanthin (Sommer, Potts, & Morrissy, 1991).

1.1.2 Culture conditions for growth and astaxanthin induction

As *H. pluvialis* is a slow-growing strain, the culture conditions such as medium, temperature, pH and light intensity must be optimized to obtain higher cell density and astaxanthin accumulation. The conditions needed for growth are different from those necessary for astaxanthin formation, which results in the need for two different stages in commercial production of astaxanthin. Different media are also used for cultivating *H. pluvialis*, BG-11 (Stanier, Deruelles, Rippka, Herdman, & Waterbury, 1979), BBM, KM1-basal medium with sodium acetate (M. Kobayashi, Kakizono, & Nagai, 1993) and OHM (Jaime Fábregas, Domínguez, Regueiro, Maseda, & Otero, 2000) are among the most popular ones. The best media composition for achieving high growth rate is different than the composition of the media that will make *H. pluvialis* cells accumulate more astaxanthin. For astaxanthin induction, there should be some stresses on microalgae. The stress can be imposed through culture media in a number of ways including nutrient deficiency in media, not having sufficient nitrogen or phosphate, adding NaCl to red stage media to increase salinity (0.25-0.5% w/v); adding ferrous sulfate to the induction media (0.45 mM) (Makio Kobayashi, Hirai, Kurimura, Ohigashi, & Tsuji, 1997; Sarada, Tripathi, & Ravishankar, 2002). The amount of astaxanthin produced by microalgae varies depending on the type and the amount of stress put on microalgae. Care is needed in the imposition of stress, as the microalgae can die before the encystment stage if the amount of stress imposed becomes higher than what they can

tolerate. The suggested temperature for growth stage is within the range of 20 to 28°C and although astaxanthin can accumulate at this temperature range, temperatures over 30°C are more suitable and accelerate the encystment process. It has been proposed by researchers that high temperature stimulates the formation of oxygen radicals and increases their reactivity and thereby helps astaxanthin production (Fan, Vonshak, & Boussiba, 1994; Kang, Han, Choi, & Sim, 2010; Tjahjono et al., 1994). pH is also an important factor affecting growth and carotenoid production. Different operating initial pHs have been studied to determine the ideal pH. Although there are some controversies in optimal initial pH among the researches, it has been reported that initial PH in the range of 6-7.5 is suitable for both green and red stages (Borowitzka, Huisman, & Osborn, 1991). Irradiation is another important parameter that must be taken into consideration for cultivating *H. pluvialis*, as it is different for the two stages of growth and astaxanthin formation. The irradiation typically used for growth stage is in the range of 40 and 50 μm photons $m^{-2}s^{-1}$ (Chekanov et al., 2014; Hata, Ogbonna, Hasegawa, Taroda, & Tanaka, 2001). High irradiation is one of the stresses that could be imposed on *H. pluvialis.* The lighting process for cultivating *H. pluvialis* is usually done by alternating cycles of light and dark, with a different cycle used for the growth and red stages. The typical cycles of light: dark used are 12:12 or 16:8 h for growth stage (Park, Choi, Hong, & Sim, 2014; Saha et al., 2013). However, it has been reported that under continuous lighting more biomass can be produced (Domínguez-Bocanegra, Guerrero Legarreta, Martinez Jeronimo, & Tomasini Campocosio, 2004). Changing the operating parameters to initiate the encystment stage can be done gradually. For instance, temperature and light intensity can be increased slowly to avoid killing the culture by giving it enough time to the culture to acclimate to the new conditions (Hata et al., 2001; Park et al., 2014). Different growth systems can be used to cultivate *H. pluvialis*, namely photoautotrophic, heterotrophic or mixotrophic.

1.1.2 Composition of *H. pluvialis*

1.1.2.1 Proteins

Astaxanthin-producing *H. pluvialis* is known for its distinct life cycle. It can be divided into two stages, the initial green stage and the secondary red stages, which have substantially different cell composition. The green stage usually has high amounts of protein, and it accounts for 29 to 45% of the total content of most *Haematococcus* strains (Shah et al., 2016). The amount of protein decreases when stress is imposed on the cells, and they start to form a cyst. Researchers have reported that the protein percentage is further reduced to 21 to 23% in the red stage which mainly consists of aspartic acid, alanine, leucine and glutamic acid (Ji Hyung Kim et al., 2015; Lorenz, 1999).

1.1.2.2 Lipids

Lipids are also a main component of *H. pluvialis*, and make up approximately 20 to 25% of the cells in the vegetative stage deposited in the chloroplast. Imposing stress on microalgae increases the lipid fraction of the cells and they can accumulate lipids up to 40% as cytoplasmic lipid droplets (Shah et al., 2016). High lipid amounts can also be achieved through imposing nutrient deficiency on the culture. Studies show that the lipid profile can be manipulated depending on the culture environment, type and amount of stress imposed, operating conditions, nutrient starvation and strain of microalgae used. *H. pluvialis* has been considered for biodiesel production because of its high lipid amount especially under stress (Damiani, Popovich, Constenla, & Leonardi, 2010; Saha et al., 2013).

1.1.2.3 Carbohydrates

Carbohydrate is also found in *H. pluvialis*. In the green stage, cells usually contain 15-17% carbohydrate (Shah et al., 2016). As the conditions become more unfavorable, carbohydrate content within the cells increases to more than double the amount found in the green stage. Different amounts of carbohydrate have been reported in cells, namely 38, 60 and 74%. Carbohydrate in the cells, starch, is consumed when high stress on *H. pluvialis* continue for a long time (Boussiba & Vonshak, 1991; Lorenz, 1999; Recht, Zarka, & Boussiba, 2012).

1.1.2.4 Carotenoids

Carotenoids are naturally occurring pigments that are essential for photosynthetic organisms. These compounds are highly valued because of their unique and various biological characteristics, high antioxidant activity, light quenching function, use as a precursor of vitamin A, and use as visually attractive pigments (Schroeder & Johnson, 1995). The carotenoid profile of *H. pluvialis* varies significantly in its two stages. In the green stage, the main carotenoids are Lutein (75-80%) and β-carotene (10-20%), as well as chlorophyll a and b, violaxanthin, neoxanthin, lactucaxanthin and zeaxanthin which contribute to a small fraction of cells (Renstrøm, Borch, Skulberg, & Liaaen-Jensen, 1981). Transition to the red stage is accompanied by an increase in carotenoid level within the cells. The carotenoids existing in the green or vegetative stage are called primary carotenoids which are replaced by secondary carotenoids in the red stage. The main fraction of carotenoids in the second stage is astaxanthin which varies between 80 to 99% of total carotenoid (Harker, Tsavalos, & Young, 1996).

Carotenoids are hydrocarbons with C_{40} polyene chain backbones. Based on the type of terminals and molecules that they have, carotenoids are divided into two groups: carotenes with hydrocarbon terminals, which have carbon and hydrogen in their molecular structure and xanthophylls with oxygenated terminals and which have oxygen in addition to carbon and hydrogen (Paliwal et al., 2016). Oxygen can be in the form of -OH group, zeaxanthin, or it can be present as oxi-groups, canthaxanthin, or a combination of both of them, astaxanthin (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006). The polyene chain and other structural characteristics can affect the functions that carotenoids have, such as their free radical scavenging power. It can also affect their location and orientation within the lipid droplets (El-Agamey et al., 2004). Carotenoids have two different geometric isomers: cis and trans isomers. Irradiation, heat and chemical reaction can change the isomers. For instance, cooking vegetables can change the isomers of the carotenoids existing in that vegetable. There has been extensive research on the effect of carotenoids on the human body and researchers have found that they are essential for human health. These compounds can protect health in the case of some chronic diseases such as cancer and heart disease (Jomova & Valko, 2013).

However, the human body does not produce carotenoids and so it must obtain it from other sources.

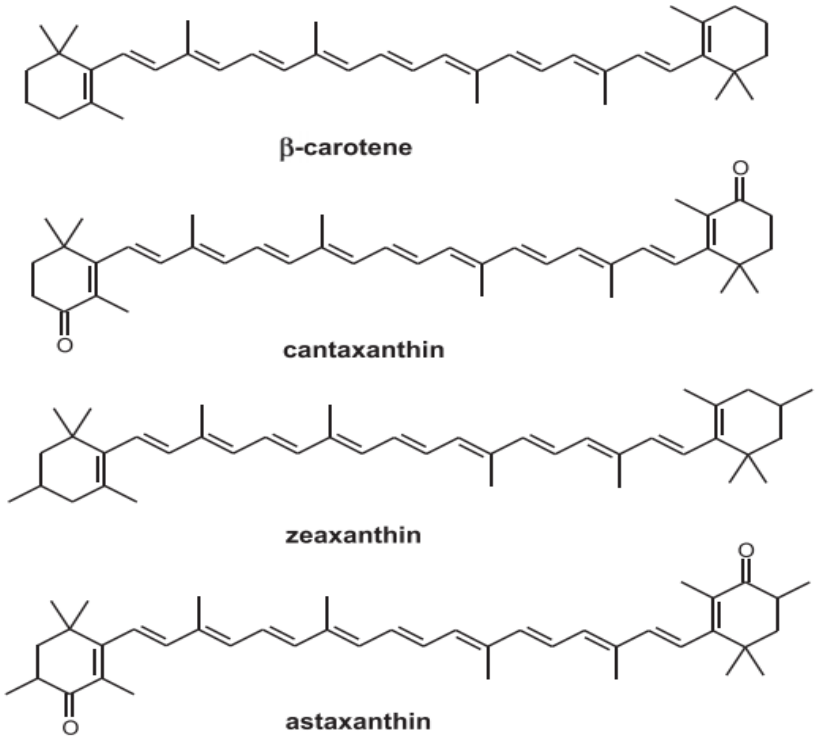

Figure 2: molecular structure of some of the carotenoids (Liu et al., 2014)**.**

Oxidation, reduction, hydrogen abstraction and addition are the main four mechanisms that are involved in the of quenching free radicals by carotenoids.

$$Car + R^{\bullet +} \longrightarrow Car^{\bullet +} + R \quad (1a)$$

Oxidation:

$$Car - e^{-} \longrightarrow Car^{\bullet +} \quad (1b)$$

Reduction: $$Car + e^{-} \longrightarrow Car^{\bullet -} \quad (2)$$

Hydrogen abstraction: $$Car[H] + R^{\bullet} \longrightarrow Car^{\bullet} + RH \quad (3)$$

Addition: $$Car + ROO^{\bullet} \longrightarrow [Car - ROO]^{\bullet} \quad (4a)$$

$$[Car - ROO]^{\bullet} + ROO^{\bullet} \longrightarrow ROO - Car - ROO \quad (4b)$$

Reaction (4b) is also called as a radical chain-breaking mechanism (Jomova & Valko, 2013).

Carotenoids can also be produced synthetically. One example is the industrial production of β-carotene. This carotenoid has 40 carbon atoms in its molecular structure. The industrial production methods can be divided into two categories based on the reactants used: symmetrical methods, number of carbons in the reactants are symmetric, and unsymmetrical ones in which molecules used as reactants do not have symmetric carbon numbers:

Symmetrical method	**Unsymmetrical method**
$\boldsymbol{C_{18} + C_4 + C_{18}}$	$\boldsymbol{C_{22} + C_{18}}$

Both of the most popular industrial methods used by companies, Badische Anilin- & Soda- Fabrik (BASF) and Roche, are symmetrical methods. BASF is based on the Witting reaction and it uses two reactants with 20 carbons:

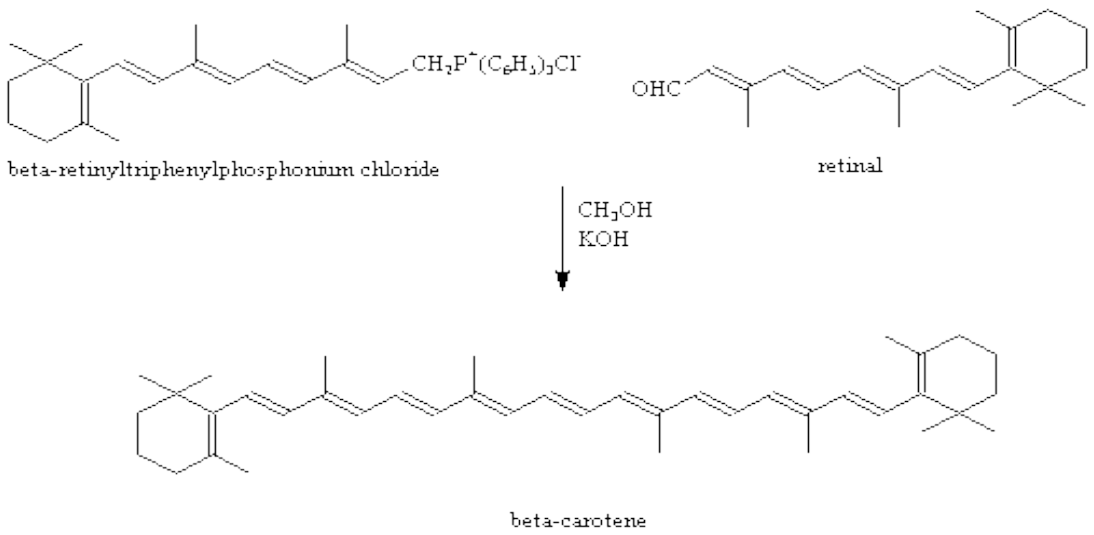

Figure 3: Badische Anilin- & Soda- Fabrik (BASF) pathway for β-carotene (Gong & Bassi, 2016)**.**

Roche is based on the Gringard reaction and it uses two molecules with 19 carbons and another one with 2 carbons to reach the 40 carbons required for β-carotene:

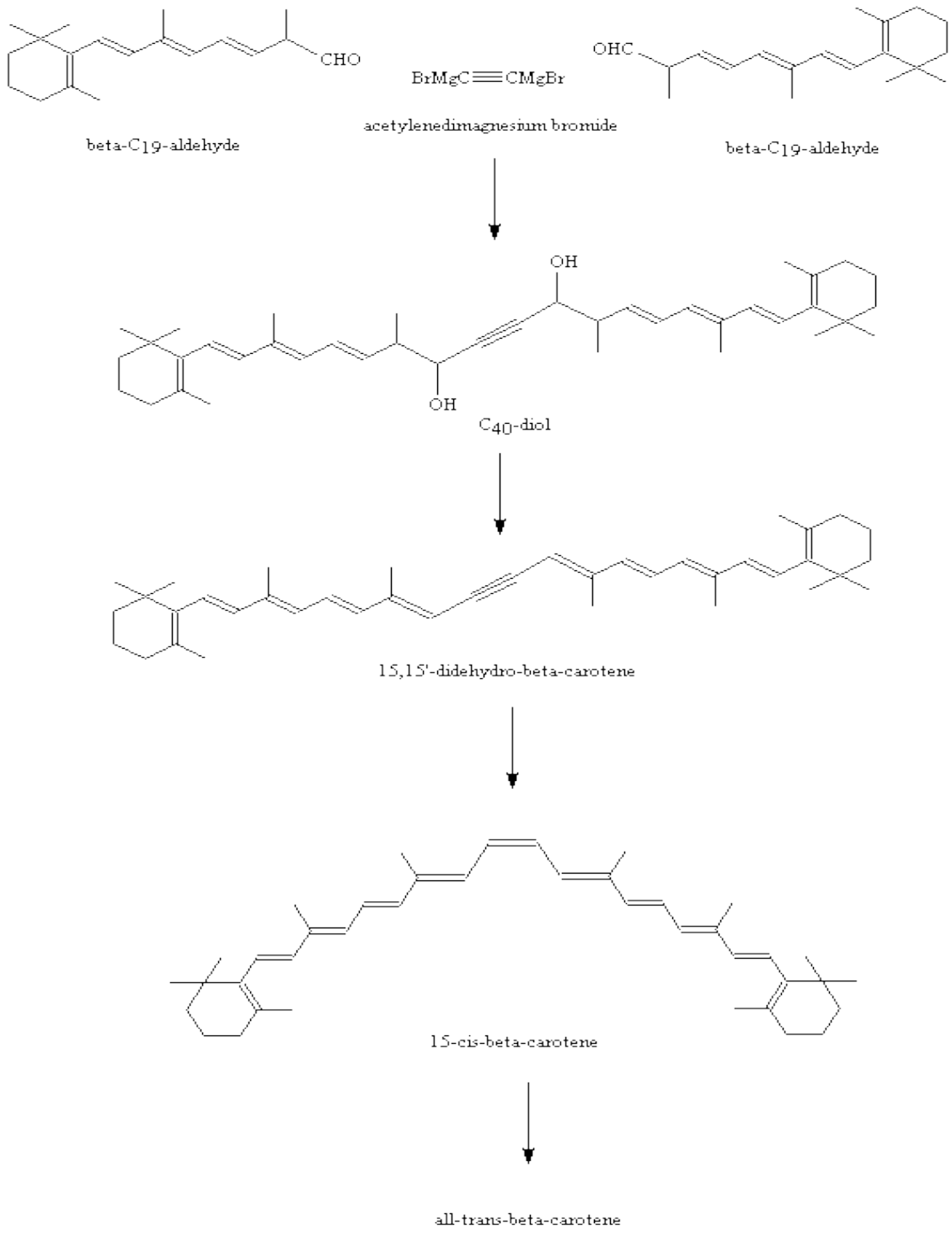

Figure 4: Roche method for β-carotene synthesis by F. Hoffman-La Roche & Co. Ltd (Gong & Bassi, 2016)**.**

1.2 Astaxanthin

Astaxanthin, 3, 3′- dihydroxy-β, β′- carotene-4, 4′- dione, is a naturally occurring xanthophyll carotenoid. It is found in different marine animals such as salmon, shrimp, trout and lobster and gives them their bright pink color. It can also be found in microorganisms like algae and yeast. Astaxanthin has shown various potential health benefits such as anti-inflammatory, anticancer and anti-aging effects. Due to its multiple applications, researchers have recently made attempts to find more cost-effective and environment-friendly methods for astaxanthin production. Researchers and many members of the public believe that astaxanthin is one of the potent natural antioxidants and has higher antioxidant activity compared to other carotenoids like lutein, α-carotene, β-carotene, and lycopene (Naguib, 2000). *H. pluvialis*, a green freshwater microalgae, is one of the main sources for natural astaxanthin production and produces the compound in greater amounts when it is under stress conditions like high irradiation, high salinity, high temperature and nutrient starvation. This microalga is used as a feed for salmon and other marine animals (Sarada et al., 2002).

1.2.1 Astaxanthin molecular structure

Astaxanthin contains carbon, hydrogen and oxygen atoms in its structure and because of the presence of oxygen is classified in xanthophyll group of carotenoids. Its molecular formula is $C_{40}H_{52}O_4$ and has a molecular weight of 596.84 g/mol. Astaxanthin has two terminal rings with hydroxyl (-OH) groups at each end of its structure and also has symmetric carbon atoms at 3 and 3′ positions of the rings. Astaxanthin exists in different forms: stereoisomers, geometric isomers, free, and esterified, all of which forms are available from natural sources (Higuera-Ciapara et al., 2006). Astaxanthin can be found in two esterified forms, di-ester and mono-ester. If one of the hydroxyl groups at the end of the ring reacts with fatty acids such as palmitic, oleic, linoleic, etc. , it becomes a mono-ester and in case that both of the rings react with fatty acids, it becomes a di-ester (Ambati, Moi, Ravi, & Aswathanarayana, 2014). (3S, 3′S) and (3R, 3′R) isomers are the most common forms of astaxanthin found in nature. *Haematococcus* accumulates more of

(3S, 3′S) isomer and *Xanthophyllomyces dendrorhous* yeast biosynthesizes more of (3R, 3′R) isomers.

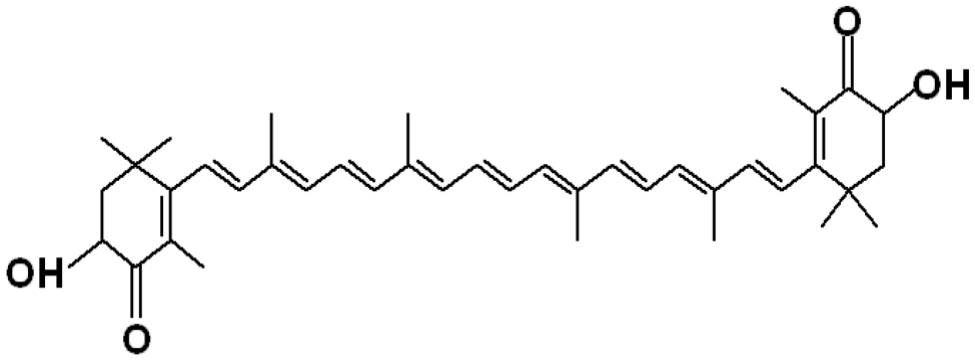

Figure 5: Molecular structure of astaxanthin (Ambati et al., 2014)

Synthetic astaxanthin has (3R, 3′R), (3S, 3′S) and (3R, 3′S) isomers with the ratio of 1:1:2 and is in the free form (Higuera-Ciapara et al., 2006).

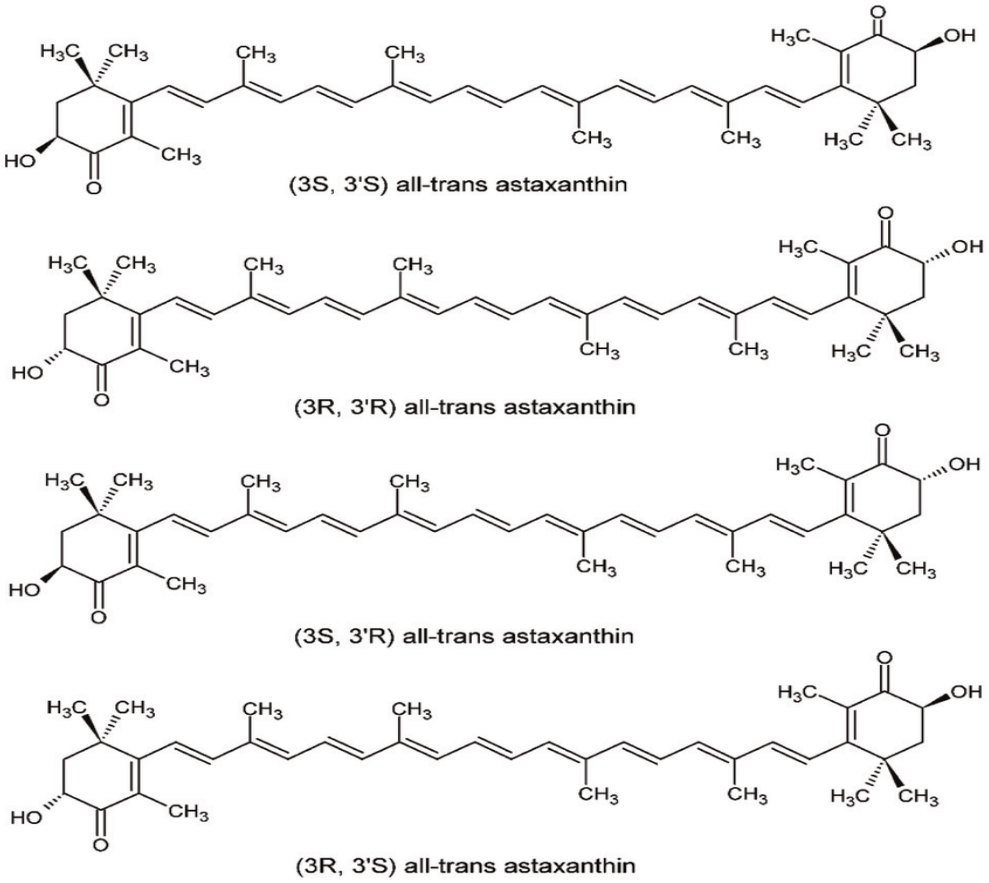

Figure 6: Different configurational isomers of astaxanthin (Khalid & J. Barrow, 2018)

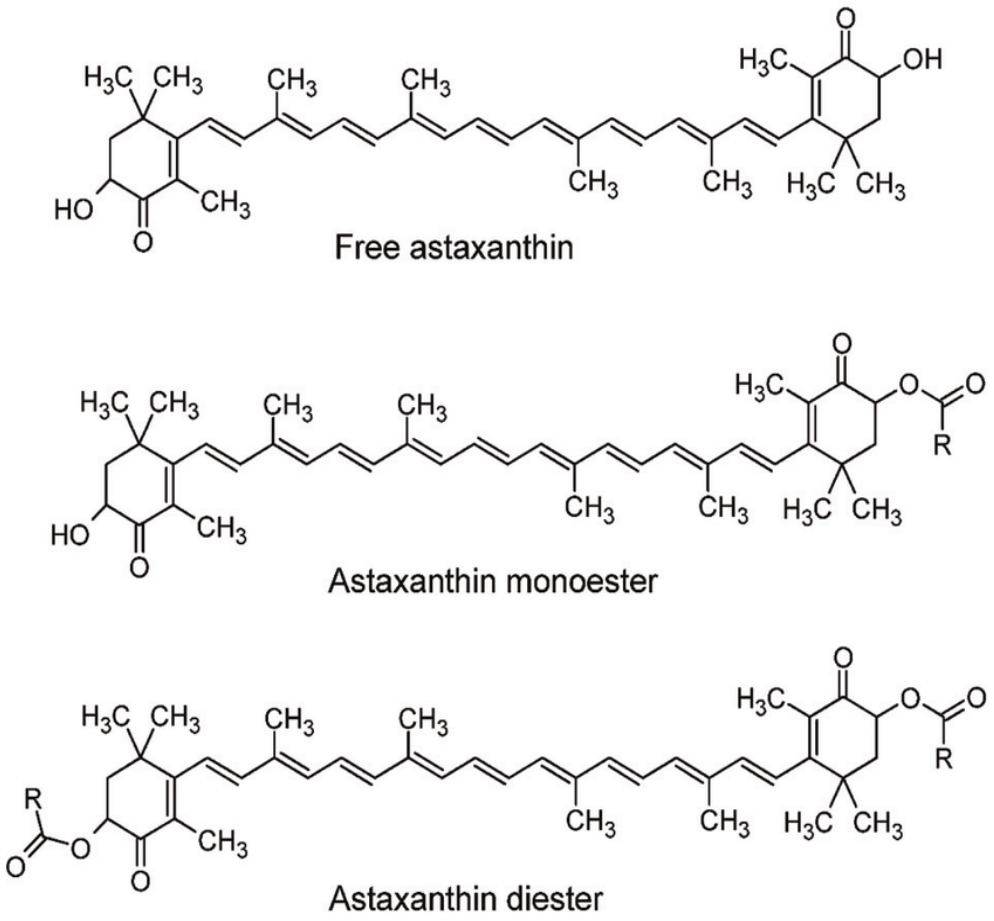

Figure 7: Free, monoester and diester forms of astaxanthin (Khalid & J. Barrow, 2018)**.**

1.2.2 Biochemistry of astaxanthin

Astaxanthin is a complex molecule and it has conjugated double bonds, hydroxyl and keto groups in its structure which give it unique characteristics such as high antioxidant activity. The red color of this pigment is due to the conjugated bond at the center of it. Astaxanthin has both lipophilic and hydrophilic properties (Higuera-Ciapara et al., 2006). Astaxanthin has a high antioxidant activity owing to the conjugated double bonds that react with free radicals and turn them into more stable products. The substance also has the ability to link with the cell membrane from inside to outside, which also accounts for its high biological activity (Guerin, Huntley, & Olaizola, 2003).

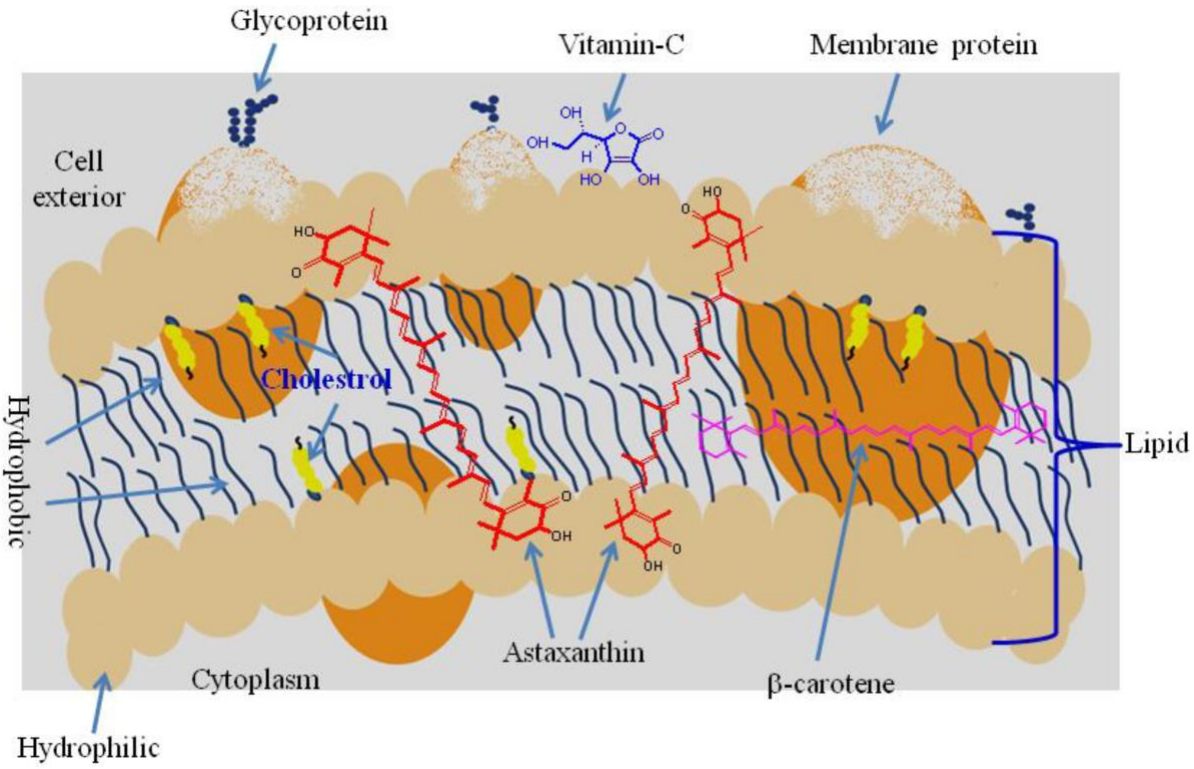

Figure 8: Astaxanthin position in the cell membrane (Ambati et al., 2014)**.**

1.2.3 Astaxanthin sources

Astaxanthin sources can be divided into two major groups, synthetic sources and natural ones. Natural sources include algae, yeast, shrimp, and trout. The major sources of astaxanthin used for commercial production are *H. pluvialis, phaffia* yeast and synthetic astaxanthin, with synthetic astaxanthin being the more significant commercially marketed astaxanthin source, with the total value more than $200 million, equal to 130 metric tons of product per year being used(J. Li, Zhu, Niu, Shen, & Wang, 2011). Until recently, synthetic versions were used instead of natural astaxanthin, but due to the growing preference for natural astaxanthin and awareness of its beneficial effects, the demand for astaxanthin extracted from *H. pluvialis* in the global market has increased. The global market for both synthetic and natural sources of astaxanthin has been estimated at 280 metric tons valued at $447 million in 2014. The market has been predicted to be $670 metric tons with the value exceeding $1.1 billions by 2020 (Shah et al., 2016).

Table 1: microorganism sources of astaxanthin (Ambati et al., 2014)**.**

sources	Astaxanthin Dry Weight Basis	References
Chloroyceae		
Haematococcus pluvialis	3.8	(Ranga Rao, Raghunath Reddy, Baskaran, Sarada, & Ravishankar, 2010)
Haematococcus pluvialis (K-0084)	3.8	(Kreuter, 1996)
Haematococcus pluvialis (Local isolation)	3.6	(Torzillo, Goksan, Faraloni, Kopecky, & Masojídek, 2003)
Haematococcus pluvialis (AQSE002)	3.4	(Olaizola, 2000)
Haematococcus pluvialis (K-0084)	2.7	(J. Wang, Han, Sommerfeld, Lu, & Hu, 2013)
Chlorococcum	0.2	(D. H. Zhang & Lee, 1997; D. H. Zhang, Lee, Ng, & Phang, 1997)
Chlorella zofingiensis	0.001	(Y. Wang & Peng, 2008)
Neochloris wimmeri	0.6	(Orosa, Torres, Fidalgo, & Abalde, 2000)
Ulvophyceae		
Enteromorpha intestinalis	0.02	(Banerjee, Ghosh, Homechaudhuri, & Mitra, 2009)
Ulva lactuca	0.01	(Banerjee et al., 2009)
Florideophyceae		
Cantenella repens	0.02	(Banerjee et al., 2009)
Alphaproteobacteria		
Agrobacterium aurantiacum	0.01	(Yokoyama, Izumida, & Miki, 1994)
Paracoccus carotinifaciens (NITE SD 00017)	2.2	(Efsa, 2005)
Tremellomycetes		

Xanthophyllomyces dendrorhous (JH)	0.5	(Aquilina et al., 2007)
Xanthophyllomyces dendrorhous (VKPM Y2476)	0.5	(Jeong Hwan Kim & Chang, 2006)
Labyrinthulomycetes		
Thraustochytrium sp. CHN-3 (FERM P-18556)	0.2	(de la Fuente et al., 2010)
Malacostraca		
Pandalus borealis	0.12	(Efsa, 2005)
Pandalus clarkia	0.015	(Meyers & Bligh, 1981)

1.3 Research objectives

The overall objective of this study was to investigate the extractability of astaxanthin from *H. pluvialis* using deep eutectic solvents in one step.

1.3.1 Specific objectives

- Assess the ability of deep eutectic solvents to disrupt the cell wall of *H. pluvialis* and reduce the number of extraction steps.
- Evaluate the extractability astaxanthin using deep eutectic solvent
- Investigate the effect of temperature on the extraction efficiency
- Study the effect of DES: water ratio on the extraction process
- Monitor how HBD: HBA molar ratio affect the astaxanthin recovery
- Optimize astaxanthin recovery using CCD

Chapter 2

2 Literature review

2.1 Astaxanthin extraction

There are two main parameters that should be considered in regards to astaxanthin recovery from *H. pluvialis*: cell disruption and astaxanthin extraction. The tough sporopollenin cell wall of *H. pluvialis* is one of the most challenging steps in astaxanthin extraction (Shah et al., 2016). Current cell disruption techniques and extraction methods will be reviewed in this chapter. Studies that used deep eutectic solvents either as extraction solvents or as pretreatment are also reviewed.

2.1.1 Cell disruption techniques

Although *H. pluvialis* is one of the main sources for astaxanthin production, the three-layered cell wall composed of cellulose and sporopollenin that characterizes its red stage prevents astaxanthin extraction and impacts bioavailability (Hagen et al., 2002). *H. pluvialis* is less permeable and more resistant to mechanical methods due to the composition of its cell wall (Machado et al., 2016). As a result of this resistance, the extraction step can be a cost and energy intensive operation (Sun, Guan, Kong, Geng, & Wang, 2016). Because of the valuable properties of astaxanthin, different physical and chemical methods have been tested to improve cell disruption to make extraction more efficient (Desai, Streefland, Wijffels, & Eppink, 2016; Machado et al., 2016; Mendes-Pinto, Raposo, Bowen, Young, & Morais, 2001).

Kobayashi et al. have reported that 70 % of the total astaxanthin available in the *H. pluvialis* cells can be extracted when cells are treated with 40% (v/v) acetone for 2 min at 80°C. It also needs another step of lyophilization or lytic enzyme treatment. They also found that without treating with heat and acetone, lyophilization or enzymes did not increase extraction efficiency (M Kobayashi et al., 1997).

Mendes-Pinto et al. evaluated the efficiency of different cell disruption methods in 2001. They reported the efficacy of autoclaving, spray drying, enzymatic treatment and

mechanical methods and also exposed the *H. pluvialis* to alkali and acidic environments. The resulting data suggested that autoclaving, mechanical methods, and spray drying were the most efficient disruption methods, respectively, and that they did not affect the total carotenoid amount. The research also indicated that using enzymatic treatments and exposing the cells to extreme pH resulted in a 20-35% loss of total carotenoid.

In another study carried out by Safi et al., four different types of cell disruption methods were tested on different microalgae. The efficacy of manual grinding, ultrasonication, chemical homogenization, and high-pressure homogenization were tested by comparing the amount of protein extracted compared to the total amount of protein available. The researchers found that *H. pluvialis* had the lowest protein recovery in all four methods in comparison with other microalgae and attributed this to its cell wall structure. Among the methods investigated, high-pressure homogenization had the best performance and after that chemical method using NaOH and HCl had a good protein recovery (Safi et al., 2014).

Machado et al. have studied the effect of three enzymes on cell disruption and extraction of astaxanthin from *H. pluvialis* by using different relative lytic activities of Glucanex®, Lyticase® and Driselase® in the absence and presence of ultrasound as disruption techniques. The researchers compared the number of total carotenoids extracted to the total carotenoids available as a measure of performance. They reported that enzymatic lysis associated with ultrasound is an efficient cell disruption method and up to 85% of carotenoid can be extracted using Glucanex® (Machado et al., 2016).

A study carried out by Ruen-ngam et al., also investigated novel cell disruption technologies to extract bioproducts from microalgae. As a part of this research, they have studied ultrasonic bath and microwave accelerated cell disruption methods followed by solvent extraction and compared the extraction amounts of this method with the astaxanthin extracted by conventional solvent extraction. It has been reported that microwave assisted method and ultrasound method had a better efficiency and up to 75% of astaxanthin was extracted followed by solvent, acetone, extraction (Ruen-ngam et al., 2010).

Sarada et al. have tested the efficiency of different pretreatments on cell disruption. The study evaluated the effects of various pretreatments using different solvents and mineral and organic acids in different treatment time, temperature, and acid concentrations on astaxanthin recovery were evaluated. They have used acetone for the solvent extraction step following the pretreatment. The results show that the treatment done using Hydrochloric acid has a higher efficiency, up to 94% than other methods (Sarada et al., 2006).

A recent study done by Desai et al. investigated the pretreatment effect of ionic liquids (IL) on permeabilizing *H. pluvialis*. They have used imidazolium, ammonium and phosphonium-based ILs and evaluated their performance in different temperatures and IL concentrations. Ethyl acetate was used for astaxanthin extraction from IL-treated cells. The results showed that among the operating temperatures, 25 °C, 45 °C, 55 °C and 65 °C, tested, 45 °C had a better impact on enhancing cell permeability. They also found out that microalgae treated with imidazolium-based ILs with 80% (w/w) concentration released more astaxanthin to ethyl acetate extraction (Desai et al., 2016).

2.1.2 Astaxanthin extraction methods

Biological active compounds from natural sources such as microalgae have attracted researchers attention because of their various practical applications (Metting & Pyne, 1986). Astaxanthin derived from *H. pluvialis*, for example, can be used as a nutritional supplement, aquaculture feed and anticancer agent to name a few (Ambati et al., 2014). Astaxanthin is a lipid-soluble compound and solvents and oils can dissolve it. There are different methods for astaxanthin extraction using solvents, edible and vegetable oils, supercritical carbon dioxide (SC-CO_2) and acids. Solvent extraction and supercritical carbon dioxide (SC-CO_2) are among the most popular extraction methods and are used widely (Shah et al., 2016).

Kang & Sim have investigated direct extraction of astaxanthin from *H. pluvialis* using different vegetable oils including soybean oil, corn oil, grapeseed oil, and olive oil. The extractions were carried out at room temperature. The reports indicated that after 48 h of extraction associated with vigorous stirring, 87.5% of available astaxanthin was released

to vegetable oil phase due to the lipophilic characteristic of astaxanthin (Kang & Sim, 2008).

Supercritical carbon dioxide (SC-CO_2) extraction has many advantages over other methods and is one of the common extraction methods used for industrial applications. Due to the low critical temperature of this process, it prevents heat-sensitive products from being degraded. Bustamante et al. have investigated astaxanthin extraction by the SC-CO_2 method using ethanol as a co-solvent. To this end, extraction was done in the temperature range of 313-343 K and pressure range of 30-55 MPa in the presence and absence of ethanol. The results show that pressure had a positive impact and temperature had a negative effect on the recovery of astaxanthin. They also reported that presence of ethanol, 4.5% (v/v), in the process increased recovery and they could extract 84% of available astaxanthin (Bustamante et al., 2011). A significant number of studies have investigated astaxanthin extraction using SC-CO_2 (Fujii, 2012; Krichnavaruk, Shotipruk, Goto, & Pavasant, 2008; Pan, Wang, & Chen, 2012; Reyes, Mendiola, Ibañez, & Del Valle, 2014; L. Wang, Yang, Yan, & Yao, 2012).

In a research carried out by Praveenkumar et al., ILs have been utilized as green solvents for astaxanthin extraction from *Haematococcus*. They have tested three different classes of ILs including 1-ethyl-3-methylimidazolium (Emim), 1-butyl-3-methylimidazolium (Bmim) and 1-butyl3-methylpyridinium (Bmpy) to evaluate their efficiency in astaxanthin extraction from germinated cells. In this work, the cells were germinated for 12 h and after that extraction was done by ILs by vortexing for 1 min at room temperature. To get a higher efficiency, two steps of extraction was performed. [Emim] $EtSO_4$ had a better efficiency and can extract 82% of astaxanthin in two stages of extraction (Praveenkumar et al., 2015).

Dong et al. have tested different solvents and mixtures of solvents to extract astaxanthin from *H. pluvialis*. Hydrochloric acid treatment followed by acetone extraction, a mixture of hexane and isopropanol (6:4, v/v), methanol extraction followed by acetone extraction and soy-oil extraction were all evaluated for their efficacy in astaxanthin extraction. The

hydrochloric acid treatment and acetone extraction system was found to have a better performance than other systems (Dong et al., 2014).

In another study, Zou et al. used a mixture of ethanol and ethyl acetate as the solvent for the extraction. They evaluated different ratios of ethanol: ethyl acetate, extraction temperature and other parameters on extraction efficiency and found that a ratio of 50% provided the highest efficiency. The results showed that increasing the temperature from 20 to 40 °C leads to an increase in astaxanthin recovery and a further increase in the temperature from 40 to 70 °C dropped the recovery. In this study, the solvent extraction step was done after a cell disruption step using ultrasound. They reported that using a solvent consist of 48% ethanol for astaxanthin extraction at 41.1 °C for 16 minutes gives the highest astaxanthin yield.

There are other studies that have used acetone (Ruen-ngam et al., 2010) (Sarada et al., 2006), ethyl acetate, chloroform/methanol (Desai et al., 2016), chloroform, hexane (Molino et al., 2018), dimethyl sulfoxide (Y. Li et al., 2012).

2.2 A review on deep eutectic solvents (DESs)

A DES is usually made up of two or three components which are mostly inexpensive and safe. These components are generally a quaternary ammonium salt or hydrogen bond acceptor (HBA) with metal salt or a hydrogen bond donor (HBD) that has the ability to form a eutectic mixture through hydrogen bond interactions. One of the major characteristics of DES is that the melting point of the formed mixture is lower than each component. They have a low freezing point and generally are in liquid form at temperatures lower than 150 °C.

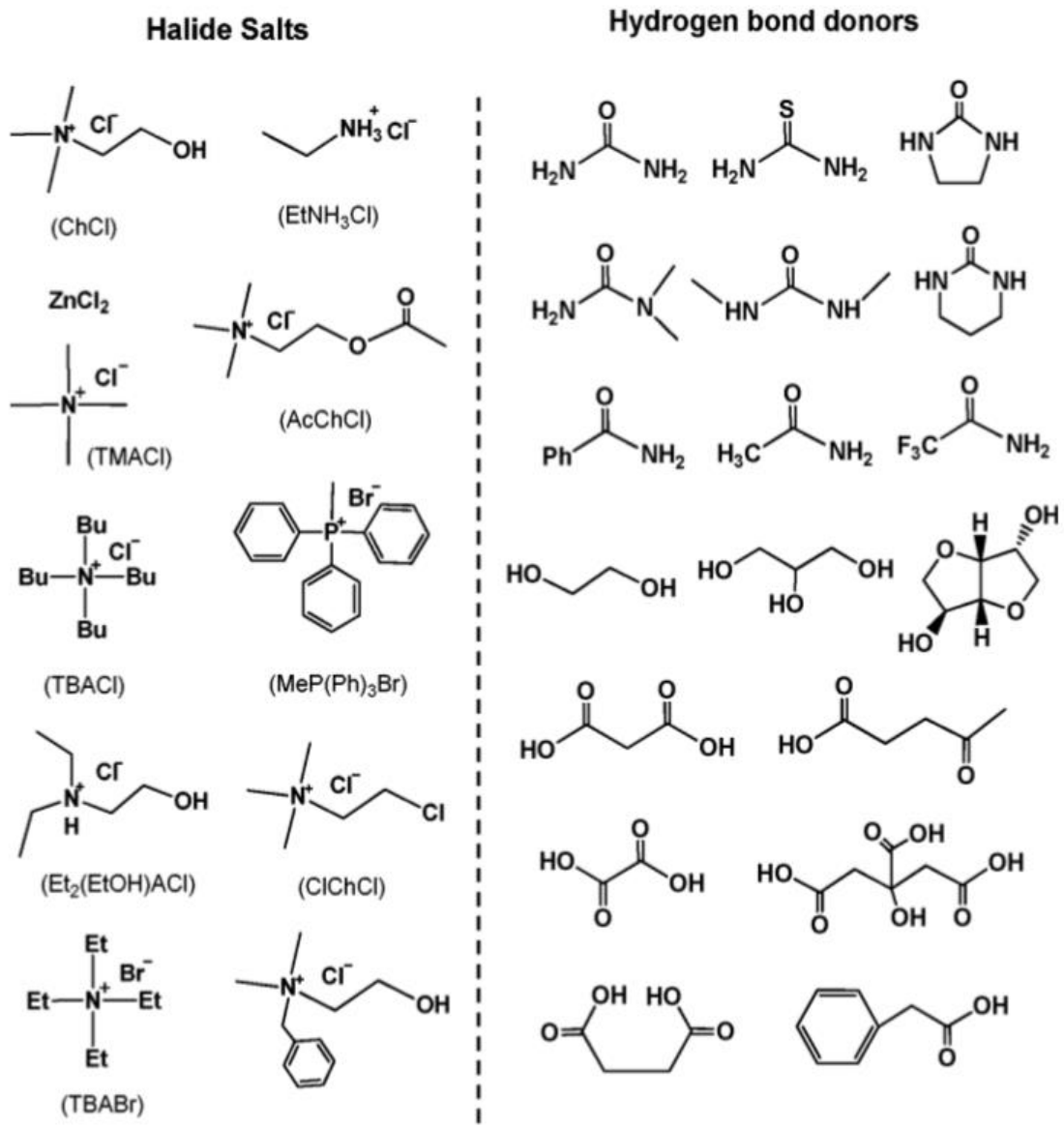

Figure 9: Structure of typical HBA and HBD used in DES synthesis (Q. Zhang, De Oliveira Vigier, Royer, & Jérôme, 2012)**.**

Choline chloride (ChCl) is one of the most popular HBAs used in DES synthesis because it is biodegradable, inexpensive and has low toxicity (Radošević et al., 2015). Urea and glycerol are also widely used HBDs. These DESs have attracted interest from researchers due to the fact that their physicochemical characteristics are similar to those of imidazolium-based ILs, but they are cheaper and safer than ILs (Degam, 2017). In comparison with conventional solvents, DESs are easier to handle and store because of their non-volatility and non-flammability (Pena-Pereira & Namieśnik, 2014).

2.2.1 Physicochemical properties of DESs

The properties of DESs such as freezing point, viscosity, density, and conductivity can be tailored by combining different quaternary ammonium salts with various HBDs. Due to their promising application in different areas, a great deal of work has been in order to characterize their properties. Here, the most important properties of DES will be discussed.

2.2.1.1 Freezing point

The two constituents of DES are linked via hydrogen bonds. The DESs formed has a lower freezing point than that of components. For example, when ChCl and urea are mixed in a molar ratio of 1: 2, the freezing point of the mixture is 12 °C while freezing point of ChCl and urea are 302 °C and 134 °C, respectively. The significant drop in freezing point can be attributed to the hydrogen bond interaction between the two components (Abbott, Capper, Davies, Rasheed, & Tambyrajah, 2003). DESs that have a freezing point lower than 50 °C get more attention from researchers since they can be used as cheap and safe solvents.

Table 2: Freezing points (T_f) of some choline-derived DESs. T_m is the melting point of pure HBDs (Q. Zhang et al., 2012)**.**

Hydrogen bond donor (HBD)	ChCl: HBD	T_m(°C)	T_f(°C)	Reference
Urea	1: 1	134	>50	(Abbott et al., 2003)
Urea	1: 2	134	12	(Abbott et al., 2003)
Ethylene glycol	1: 2	-12.9	-66	(Shahbaz, Mjalli, Hashim, & Al Nashef, 2010)
Glycerol	1: 2	17.8	-40	(Hayyan, Mjalli, Hashim, & AlNashef, 2010)
Oxalic acid	1: 1	190	34	(Abbott, Boothby, Capper, Davies, & Rasheed, 2004)
Citric acid	1: 1	149	69	(Abbott et al., 2004)

Table 2 shows the significant reduction in freezing point of DESs compared to their individual melting points. It also shows that the molar ratio of HBD: HBA has a considerable impact on the freezing point of DESs. It can be said that when the HBD ratio is increased in the mixture, the freezing point decreases.

2.2.1.2 Phase Behavior

DESs show different behavior than ideal mixtures. The significant interaction between the two components of DES, HBD and HBA, leads to a major temperature drop of the mixture and forms a eutectic point (Garc, Aparicio, Ullah, & Atilhan, 2015). The figure below is a schematic representation of the phase behavior of a mixture of the HBA and HBD.

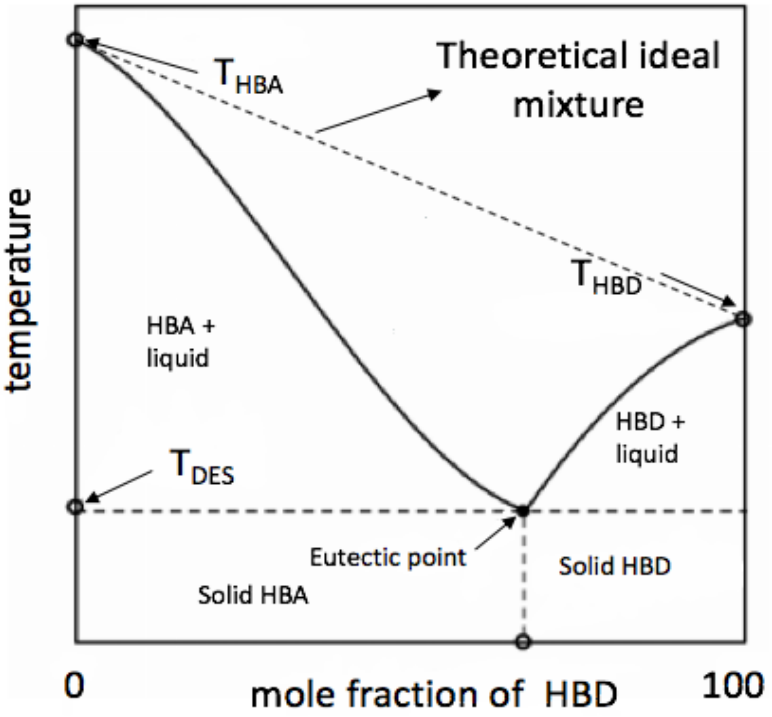

Figure 10: phase diagram of a binary mixture, HBD and HBA (Garc et al., 2015)**.**

2.2.1.3 Density

Density is another important physical characteristic for a solvent. A specific gravity meter is usually used for measuring densities of DESs. Majority of DESs are denser than water. DESs like imidazolium-based ILs have holes and empty spots, hole theory, and this could be an explanation of higher densities of DES formed compared to that of their constituents. When two components are mixed, the average hole radius decreases leading to an increase of the DES density (Abbott, Barron, Ryder, & Wilson, 2007).

Table 3: Densities of some common DESs at 25 °C (Q. Zhang et al., 2012)**.**

Salt (HBA)	HBD	HBA: HBD	Density ($g.cm^{-3}$)	Reference
$ZnCl_2$	Hexanediol	1: 3	1.38	(Abbott, Barron, et al., 2007)
$ZnCl_2$	Acetamide	1: 4	1.36	(Abbott, Barron, et al., 2007)
$ZnCl_2$	Urea	1: 3.5	1.63	(Abbott, Barron, et al., 2007)
$ZnCl_2$	Ethylen glycol	1: 4	1.45	(Abbott, Barron, et al., 2007)
ChCl	Glycerol	1: 3	1.2	(Abbott, Harris, & Ryder, 2007)
ChCl	Glycerol	1: 2	1.18	(Abbott, Harris, et al., 2007)
ChCl	Glycerol	1: 1	1.16	(Shahbaz, Baroutian, Mjalli, Hashim, & Alnashef, 2012)
ChCl	Ethylene glycol	1: 2	1.12	(Abbott, Harris, et al., 2007)
ChCl	Urea	1: 2	1.25	(Abbott, Capper, & Gray, 2006)

Table 3 lists the densities of some common DESs. The molar ratio of HBA: HBD has an impact on the densities of DES. For ChCl-G mixture, density increases by the addition of more glycerol to the system.

2.2.1.4 Viscosity

Viscosity is another significant physical property of DES and most DES have high viscosity (>100 cP) at room temperature, though there are a few exceptions. There are a number of explanations for the high viscosity of DES. The strong hydrogen bond network between two components which hinders mobility of free species within the DES is a contributing factor. Large ion size, electrostatic force and van der Waals interaction may also explain the high viscosity of DES. Temperature, components, HBD: HBA molar ratio and the water content also have a significant impacts on the viscosity of DES (Abbott et al., 2006; Q. Zhang et al., 2012).

Table 4: Viscosities of ChCl-based DESs in different temperatures (Q. Zhang et al., 2012)**.**

Salt (HBA)	HBD	HBA: HBD	Viscosities (cP)	Reference
ChCl	Urea	1: 2	750 (25 °C)	(D'Agostino, Harris, Abbott, Gladden, & Mantle, 2011)
ChCl	Urea	1: 2	169 (40 °C)	(Abbott et al., 2006)
ChCl	Ethylene glycol	1: 2	36 (20 °C)	(Abbott, Harris, et al., 2007)
ChCl	Ethylene glycol	1: 2	37 (25 °C)	(D'Agostino et al., 2011)
ChCl	Ethylene glycol	1: 3	19 (20 °C)	(Abbott, Harris, et al., 2007)
ChCl	Glycerol	1: 5	500 (25 °C)	(Abbott et al., 2011)
ChCl	Glycerol	1: 4	440 (25 °C)	(Abbott, Harris, et al., 2007)
ChCl	Glycerol	1: 3	400 (25 °C)	(Abbott, Harris, et al., 2007)

As it is shown in Table 4, temperature has a significant impact on viscosity. The table also shows that a temperature increase results in a reduction in the viscosity. Usually adding more HBD to the system decreases the viscosity but, interestingly, that does not apply for ChCl-G mixture and increasing the amount of glycerol in the system increases the viscosity. This can be explained by the partial breakdown in the strong intermolecular hydrogen bonds.

2.2.1.5 Polarity

The polarity scale, E_T (30), is a measure to determine the polarity of a solvent. E_T (30) is the electronic transition energy of a probe dye, like Reichardt's Dye 30, in a solvent (Abbott et al., 2011). E_T (30) can be calculated by the following equation:

$$\boldsymbol{E_T(30)(kcal\ mol^{-1}) = 28591/\lambda_{max}} \qquad (1)$$

Table 5: Polarity of ChCl-G mixtures with different HBA: HBD ratio (Q. Zhang et al., 2012).

Solvent	HBA: HBD	E_T (30)/kcal mol^{-1}
Glycerol	-	57.17
ChCl-G	1: 3	57.96
ChCl-G	1: 2	58.28
ChCl-G	1: 1.5	58.21
ChCl-G	1: 1	58.49

Table 5 shows that ChCl-G mixture has a higher polarity compared to the pure glycerol in any molar ratio and adding more glycerol to the system decreases solvent polarity.

2.2.1.6 Surface tension

Limited studies have been done on the surface tension of DESs. DESs have a higher surface tension compared to conventional solvents. Surface tension is strongly related to the intermolecular interaction and as a result, it is expected to have a similar trend to viscosity. The studies show that the surface tension of ChCl-G decreases as glycerol amount decreases which depends on the strong intermolecular hydrogen bond in glycerol (Abbott et al., 2011).

Table 6: surface tension values of some widely used DESs (Q. Zhang et al., 2012)**.**

Salt (HBA)	HBD	HBA: HBD	Surface tension (mN. m^{-1})	Reference
ChCl	Ethylene glycol	1: 3	45.4 (20 °C)	(Abbott, Harris, et al., 2007)
ChCl	Glycerol	1: 3	50.8 (20 °C)	(Abbott, Harris, et al., 2007)
ChCl	Malonic acid	1: 1	65.68 (20 °C)	(Abbott et al., 2004)
$ZnCl_2$	Ethylene glycol	1: 4	56.9 (25 °C)	(Abbott, Barron, et al., 2007)
$ZnCl_2$	Urea	1: 3.5	72 (20 °C)	(Abbott, Barron, et al., 2007)

2.3 DESs as extraction solvents

In recent years, DES has attracted the attention of researchers because of their potential use as sustainable and biodegradable solvents in extraction processes in different fields (Pena-Pereira & Namieśnik, 2014). DESs have also been used as a pretreatment step in different processes like algal pretreatments (Lu et al., 2016; Tommasi et al., 2017). In this section a brief review has been presented on the studies that have used DESs as astaxanthin extraction solvents and as pretreatment for microalgae.

DESs are usually formed by mixing two low toxicity and inexpensive components and are very promising extraction solvents because of their physicochemical properties. One of the more recent studies that has evaluated DES performance in the extraction of astaxanthin was carried out by Zhang et al. The study investigated the efficiency of different DES in astaxanthin extraction from local shrimp byproducts associated with the microwave cell disruption step. They have tested different choline chloride-based DES in different HBD: HBA ratios and different water contents. The reported results show that 1,2-butanediol had the highest performance among the HBDs tested. The HBD: choline chloride ratio of 5 has been reported to be the optimum ratio. The samples that contained 10% water demonstrated better efficiency in this work (H. Zhang et al., 2014).

Microalgae are a potential source for different valuable bioactive compounds. In order to extract the bioproducts from microalgae, different extraction methods have been developed. Some of the methods include one or two steps of pretreatment steps. The main goal of most pretreatments is to disrupt the cellular structure to increase the efficiency of the extraction process. Tommasi et al. have studied the effect of different DESs composed of choline chloride as HBA and various HBDs including oxalic acid, levulinic acid, urea, ethylene glycol, and sorbitol as pretreatments on lipid extraction from *phaedactylum tricornutum*. They have reported that DESs that were composed of choline chloride and carboxylic acid performed better in enhancing both the selectivity and extraction yield (Tommasi et al., 2017).

In another study, Lu et al. pretreated *Chlorella sp.* with three various DESs, choline chloride-oxalic acid (Ch-O), choline chloride-ethylene glycol (Ch-EG), and urea-acetamide (U-A). The results showed that Ch-O mixture had the highest lipid recovery, 80.90% compared with others and fatty acid profile of treated cell was same with untreated ones (Lu et al., 2016).

Chapter 3

3 Materials and methods

There are main culture media that has generally been used for the growth phase of *H. pluvialis* such as BG11, optimal Haematococcus medium (OHM), and Bold Basal medium (BBM) (Chekanov et al., 2014; J. Fábregas, Domínguez, Regueiro, Maseda, & Otero, 2000; Tripathi, Sarada, Ramachandra Rao, & Ravishankar, 1999) and also various stresses that could be imposed on the cells to start encystment phase such as intensive irradiation and insufficient nitrogen and phosphate (Chekanov et al., 2014). This section gives an overview of the materials and conditions used during the growth and red phase. After the red phase, deep eutectic solvents were used for astaxanthin extraction. The preparation and constituents of deep eutectic solvents that were used will be discussed.

3.1 Materials

Fresh green CPCC 93 *H. pluvialis* Flotow em. Wille was kindly provided by Dr. Amarjeet Bassi's lab. Astaxanthin standard (≥ 97%), Choline chloride (≥ 98%) and 1,2-butanediol (≥ 98%) was purchased from Sigma Aldrich Canada (Oakville, Ontario). Glycerol (≥ 99.9%) and was ethylene glycol (≥ 99%), purchased from Fisher Scientific Canada (Whitby, Ontario) and VWR Canada, respectively. All other chemicals used were of analytical grade.

3.2 Methods

3.2.1 Microalgae strain and growth phase conditions

H. pluvialis was grown in modified Bold's Basal Medium (BBM) (Schwartz, 2007) and sodium acetate (0.25 g/l) was also added to the media, which is detailed in Table 7. The culture was incubated at 22 °C. The light cycle used for the green stage was a light: dark (16:8 h) cycle for 10 days. Irradiance was provided by white fluorescent lamps. In this stage, air sparging was used.

Table 7: Modified Bold's Basal Medium

#	Stock	Stock solution	ml/L
1	KH_2PO_4	8.75 g/ 500 ml	10
2	$CaCl_2.2H_2O$	12.5 g/ 500 ml	1 ml
3	$MgSO_4.7H_2O$	37.5 g/ 500 ml	1 ml
4	$NaNO_3$	125 g/ 500 ml	1 ml
5	K_2HPO_4	37.5 g/ 500 ml	1 ml
6	NaCl	12.5 g/ 500 ml	1 ml
7	$Na_2EDTA.2H_2O$	10 g/ L	1 ml
	KOH	6.2 g/L	
8	$FeSO_4.7H_2O$	4.98 g/L	1 ml
	H_2SO_4 (concentrated)	1 ml/L	
9	Trace Metal Solution	Table …	1 ml
10	H_3BO_3	5.75g/ 500 ml	0.7 ml

Sodium acetate (0.25 g/L) was added to the media presented in Table 7. The initial pH of the culture was set to 6.5. Prepared media was then autoclaved and cultured with 10% (v/v) inoculation under aseptic conditions in laminar air flow hood after it was cooled down.

Table 8: Components of trace metal solution.

#	Substance	Per Littre
1	H_3BO_3	2.86 g
2	$MnCl_2.4H_2O$	1.81 g
3	$ZnSO_4.7H_2O$	0.222 g
4	$Na_2MoO_4.2H_2O$	0.390 g
5	$CuSO_4.5H_2O$	0.079 g
6	$Co(NO_3)_2.6H_2O$	0.0494 g

Table 8 shows the components of the trace metal solution that was mentioned in Table 7.

3.2.2 Culture conditions for red stage

In order to start the transition to the red stage, flocculation was stopped and the temperature for the incubation was increased to 28 °C. To stimulate astaxanthin biosynthesis, NaCl (100 mM) was added to the red stage media and continuous lighting was used for a period of 2 weeks. After two weeks the cells that were red due to astaxanthin accumulation were harvested by centrifugation at 3500 rpm for 10 min at 4 °C in a Sorvall R40 centrifuge (Thermo-Scientific, USA).

Figure 11: Pictures of *H. pluvialis* in green and red stage.

As Figure 11 shows, the algae are dark red, which indicates the accumulation of astaxanthin in the cells. Aeration has been removed from the system and cells are settled in the red stage.

3.2.3 Freeze Drying

Water was removed from centrifuged cells through a freeze dryer in order to prepare cells for extraction. To this end, microalgae were washed three times with deionized water and frozen at -80 °C overnight. The harvested biomass was lyophilized using 4.5 L freeze-drier (Labconco) for 24 h. After freeze-drying microalgae were ready to be used for extraction. In order to prevent astaxanthin degradation, dried *H. pluvialis* was tightly sealed and covered by aluminum foiled and stored at -20 °C until use.

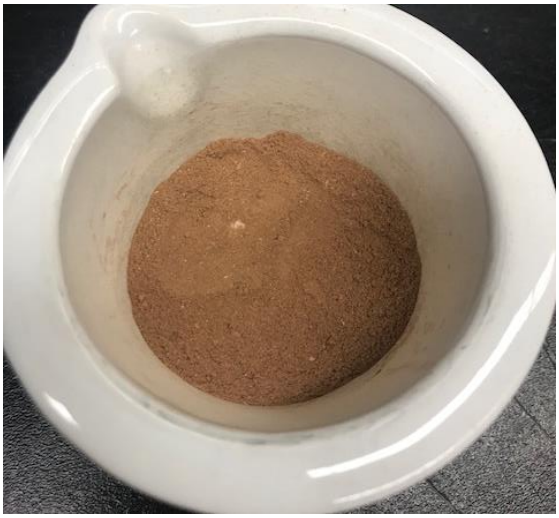

Figure 12: Powder form of freeze-dried *H. pluvialis.*

3.3 Measurement of total astaxanthin

In order to determine total astaxanthin content, 20 mg freeze-dried *H. pluvialis* cells were disrupted using mini-beadbeater (Bio spec Products Inc., USA) for 5 minutes at a speed of 4800 rpm followed by a solvent extraction using a chloroform-DMSO mixture with a volume ratio of 2: 4. After precipitation of algae, supernatant layer, chloroform-DMSO was removed to another container and was diluted with DMSO. The astaxanthin absorbance was measured by scanning the sample in a glass cuvette using a UV-Vis spectrophotometer at 472 nm wavelength (Thermo-Scientific, USA).
Total astaxanthin can be calculated using equation 2 which has been obtained using different concentrations of standard astaxanthin.

$$\boldsymbol{Total\ astaxanthin}\ (\frac{\boldsymbol{mg}}{\boldsymbol{ml}}) = \frac{[\boldsymbol{A_{472}}]-\boldsymbol{0.0169}}{\boldsymbol{180.15}} \quad (2)$$

3.4 DES preparation

Deep eutectic solvents were synthesized by heating choline chloride and the hydrogen bond donor to 80 °C until a clear homogeneous liquid was formed (Abbott et al., 2006). The mixture was stirred continuously. DESs, different HBD: HBA ratios and abbreviations that were used in this work are listed in Table 9.

Table 9: DESs used in the experiments

abbreviation	HBA	HBD	HBD: HBA
ChCl-G	Choline chloride	Glycerol	0.5
			2
			2.5
			3
			4
			4.5
			5
			6.5
			7.5
ChCl-EG	Choline chloride	Ethylene glycol	2
			3
			4
			5
ChCl-B	Choline chloride	1,2-butanediol	2
			4

3.5 Extraction procedure

The extractions were performed as follows: 20 milligrams of dried *H. pluvialis*, deep eutectic solvent and water were mixed together at the indicated ratios in vials. The vials were mixed using a magnetic micro-stirrer. The water was used as the only solvent for the blank experiment. In order to have a homogeneous heat transfer, vials were placed in a water bath at the specified temperature. Furthermore, vials were tightly sealed and covered with aluminum foil to inhibit any degradation due to air and light. For analysis, after the extraction was finished, the samples were diluted with dimethyl sulfoxide (DMSO) and centrifuged. To measure astaxanthin absorbance, samples were scanned using UV-Vis spectrophotometer at 472 nm wavelength.

3.6 Experimental design

In order to gain a better understanding of the response and be able to optimize astaxanthin recovery, the response, central composite design (CCD) was used for the design of experiments (DOE). CCD is one of the most widely used design in response surface methodology (RSM). CCD is a factorial or fractional factorial design with replicates in center point and experiments in axial points that allows for detecting curvatures (D. C. Montgomery & G.C.Runger, 1994). Temperature (*T*), the molar ratio of HBD: HBA (*M*), the volume ratio of DES: water (*V*), and time (*t*) were chosen for independent variables. The range of parameters and center point conditions were selected based on preliminary single factor experiments. To minimize the effect of unexpected variability and errors in experiments, experimental runs were randomized.

Chapter 4

4 Results and discussions

The large amounts of astaxanthin accumulated in *H. pluvialis* has been of great interest to the researchers and extensive investigation is being conducted in order to develop new and more efficient extraction methods to obtain high-quality products while being environment friendly (Desai et al., 2016; Reyes et al., 2014; Thana et al., 2008; Zou et al., 2013). DESs have gained popularity as substitutions for traditional extraction solvents because they are both cost-effective and less damaging to the environment, but their application in various fields has not been thoroughly studied (Lee & Row, 2016; X. Li & Row, 2016; Lu et al., 2016; Mbous et al., 2017; H. Zhang et al., 2014; Q. Zhang et al., 2012). These solvents have exclusive physical properties which could be adjusted by choosing different components (Mainberger et al., 2017). Other important characteristics that contribute to the growing reputation of DES are non-flammability, non-volatility, non-toxicity in many combinations, biodegradability and their low cost to name a few (Q. Zhang et al., 2012). DESs have been used as additives for the ultrasonic extraction of astaxanthin from marine plants (Lee & Row, 2016). They have also been used as solvents in an ultrasound-assisted extraction of astaxanthin from shrimp (H. Zhang et al., 2014). However, they have not been studied as solvents for the extraction of astaxanthin from *H. pluvialis.*

This study investigated the ability of DES to disrupt the thick cell wall of *H. pluvialis*. Choline chloride based DESs were tested to extract astaxanthin from *H. pluvialis*. The effect of operating parameters, temperature, molar ratio of HBD: HBA, volume ratio of DES: water, and time on the astaxanthin recovery were also studied. The central composite design was employed to detect any existing interactions among different factors and determine optimal design conditions within the ranges that experiments were designed.

4.1 *H. pluvialis* growth

In order to get a better cell density and encourage greater production of carotenoid produced, sodium acetate was added to the media. Sodium acetate is one of the important carbon sources for both the green and red stages (Borowitzka et al., 1991; Orosa et al., 2000). It inhibits growth in high concentrations, but it also helps carotenoid production and concentration adjustment is needed to achieve a balance between growth and carotenoid production (Makio Kobayashi, Kakizono, & Nagai, 1991). In this research, 0.25 g. L^{-1} sodium acetate was added to the modified BBM as an organic source due to its positive effects on both growth and encystment stages.

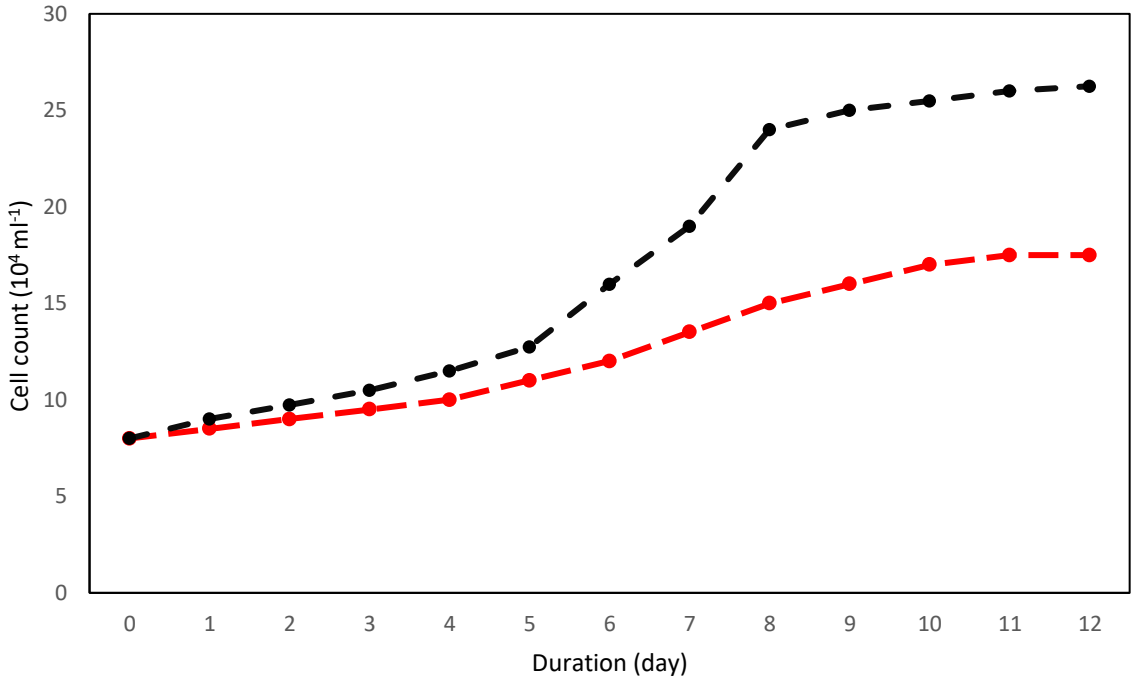

Figure 13: Effect of adding sodium acetate to modified BBM monitored in 12 days. The black line shows the cell density of *H. pluvialis* that sodium acetate was added to its media. Red line shows the cell number for microalgae cultured with BBM.

Figure 13 shows the effect of adding 0.25 g. L^{-1} sodium acetate to the growth media with initial pH 6.5. It also shows that higher cell counts can be achieved in a shorter period

when the media contains a low amount of sodium acetate. The cell number achieved is comparable with

4.2 Astaxanthin extraction from *H. pluvialis*

Various methods for improving the extraction efficiency of astaxanthin from *H. pluvialis* have been studied. The most significant issue faced when extracting astaxanthin from this microorganism is its tough cell wall, which is resistant to breakage. The majority of the extraction processes are associated with a cell disruption step are very energy consuming and expensive. In this research, DESs were used as extraction solvents and extraction was carried out in one step.

4.2.1 Astaxanthin extraction using DES

The solubility of chemicals in DESs depends on the constituents of DES and the ratio of hydrogen bond donor in the mixture (H. Zhang et al., 2014). DESs with low polarity are reported to be more compatible with chemical structure and properties of astaxanthin. Different choline chloride-based DESs composed of polyol HBDs were selected because of their low polarity to evaluate their ability to permeabilize *H. pluvialis* and extract astaxanthin simultaneously.

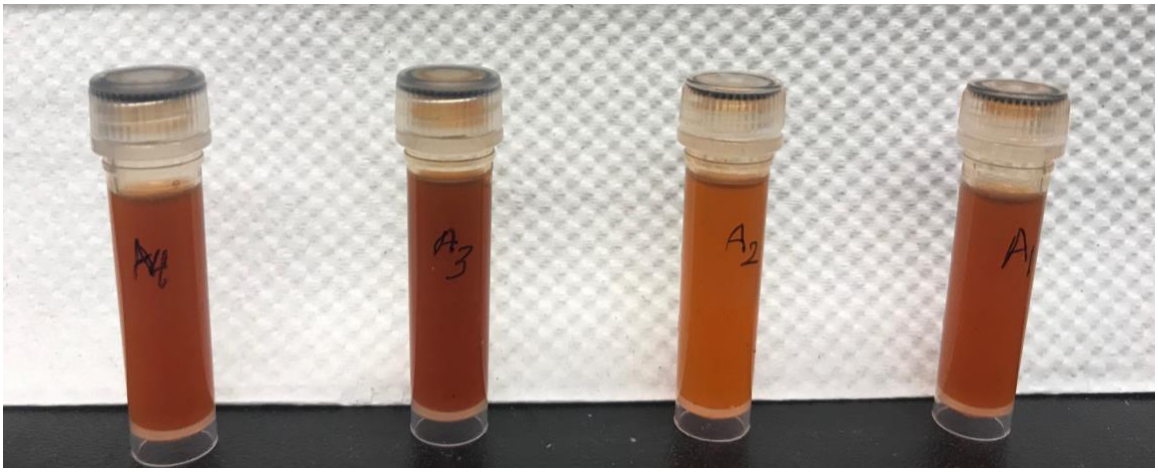

Figure 14: Extracts of the experiment after centrifugation.

Figure 14 shows the extracts after being centrifuged. Red color in the solution is an indication of astaxanthin presence in the samples. In order to investigate the effect of

DESs on the cell wall of *H. pluvialis*, microalgae cells after extraction have been observed under microscope.

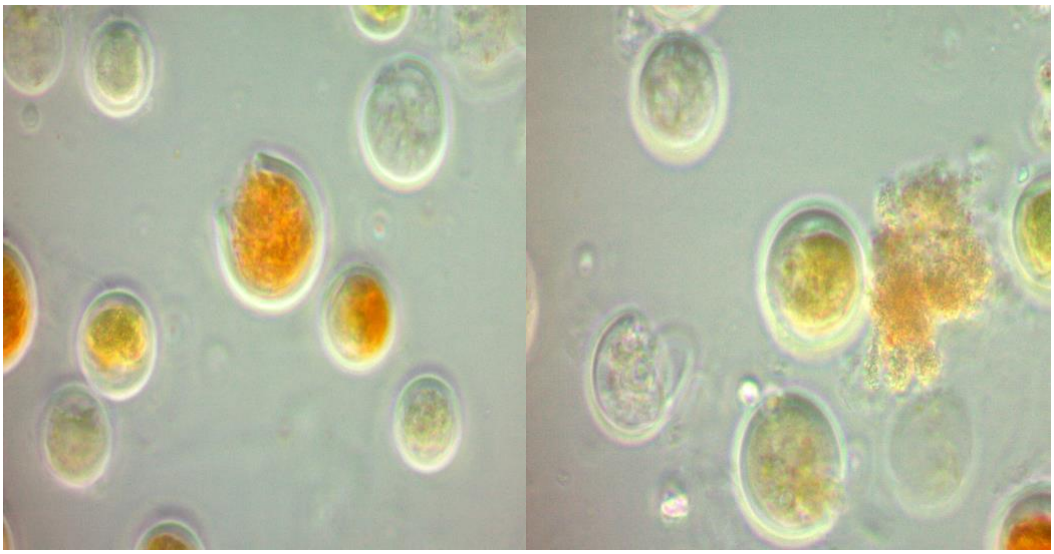

Figure 15: Microscopic view of the cells after extraction with DES- 100x magnification.

As it can be seen in Figure 15, DESs had the ability to disrupt the cell wall of *H. pluvialis* and extract astaxanthin.

4.2.1.1 Effect of DES components on extraction

Based on the characteristics and solubility of astaxanthin, preliminary tests were carried out for three different DESs, ChCl-B, ChCl-G and ChCl-EG. Considering the conditions that were used for extraction of astaxanthin using DES, a molar ratio of HBD: HBA was set to 4, the temperature that experiments were carried out was set to 60 °C and 7 h was the duration of extraction. Other co-solvents like acetone and methanol were tested in the event that they were more suitable than water, and while the data is not shown, but water was found to be easier to handle and analyze, more compatible with DES and astaxanthin, and cheaper than the alternatives.

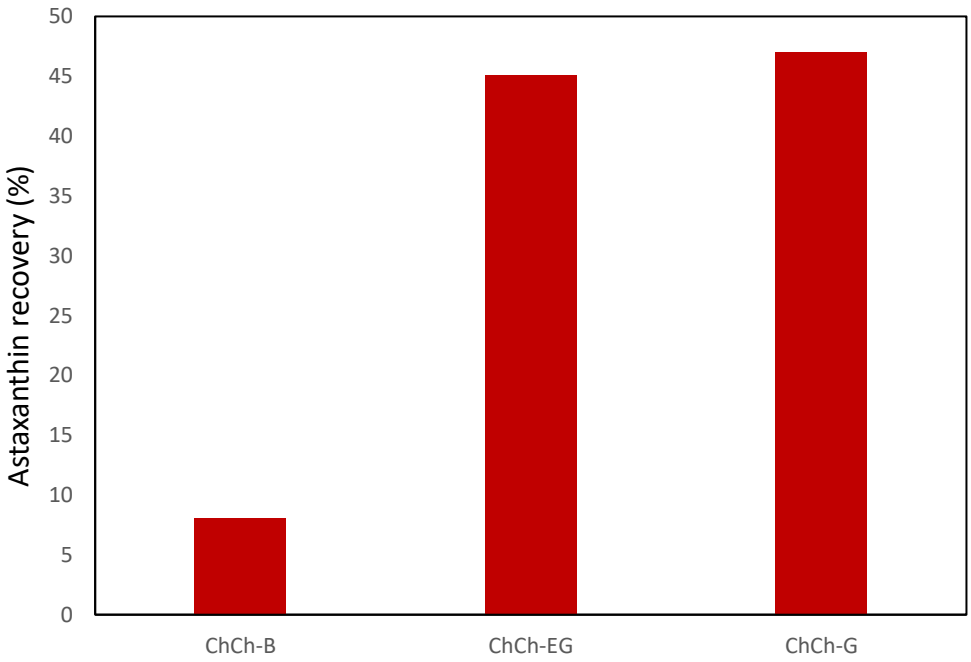

Figure 16: Astaxanthin recovery. ChCl-G had the highest extraction (47%) efficiency among other DESs tested. HBD: HBA is 4.

As is shown in Figure 16, the mixture of ChCl-B was not successful in extracting astaxanthin. In a study carried out using DESs for astaxanthin extraction from shrimp byproducts by ultrasound-assisted method, the mixture of ChCl-B had the highest efficiency (H. Zhang et al., 2014). The comparison between the outcome of the H. Zhang et al. study and these experimental results demonstrates a discrepancy that can be attributed to the cell wall disruption step. The other study used ultrasonication for

disrupting the cell wall, while this study did not. Viscosity, polarity and surface tension are among the properties that have the most impact on the extraction efficiency. Another parameter that affects extraction is the ability of DES to permeate the cell wall, which depends on the components of DES. In this study, direct astaxanthin recovery was investigated and as a result, permeabilizing and extraction cannot be interpreted independently. The DESs, ChCl-G had a slightly higher astaxanthin recovery than ChCl-EG.

4.2.1.2 Effect of water on extraction efficiency

Water content is one of the factors that affects some of the physical properties of DESs such as density, viscosity, and surface tension. Astaxanthin extraction efficiency is in turn influenced by these physical properties. Addition of water to the system decreases viscosity and density and lower viscosity helps extraction by increasing the movement of free species in the system. However, when the water content is too much, it results in an increase in solvent polarity and decrease hydrogen bonds number in the system.

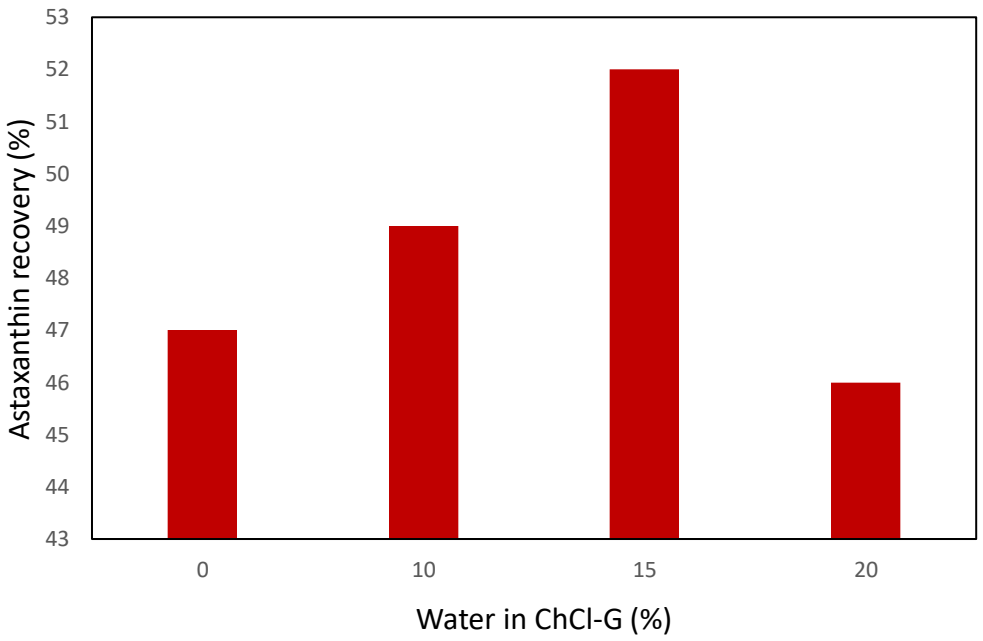

Figure 17: Effect of water content in DES on astaxanthin extraction. The system containing 15% water gave better results than others tested.

4.2.1.3 Effect of HBD: HBA ratios on astaxanthin recovery

Physicochemical properties of DES are a function of different molar ratios of HBD: HBA. To investigate the effect of various molar ratios of HBD: HBA, experiments were carried out using ChCl-G as an extraction solvent in different ratios.

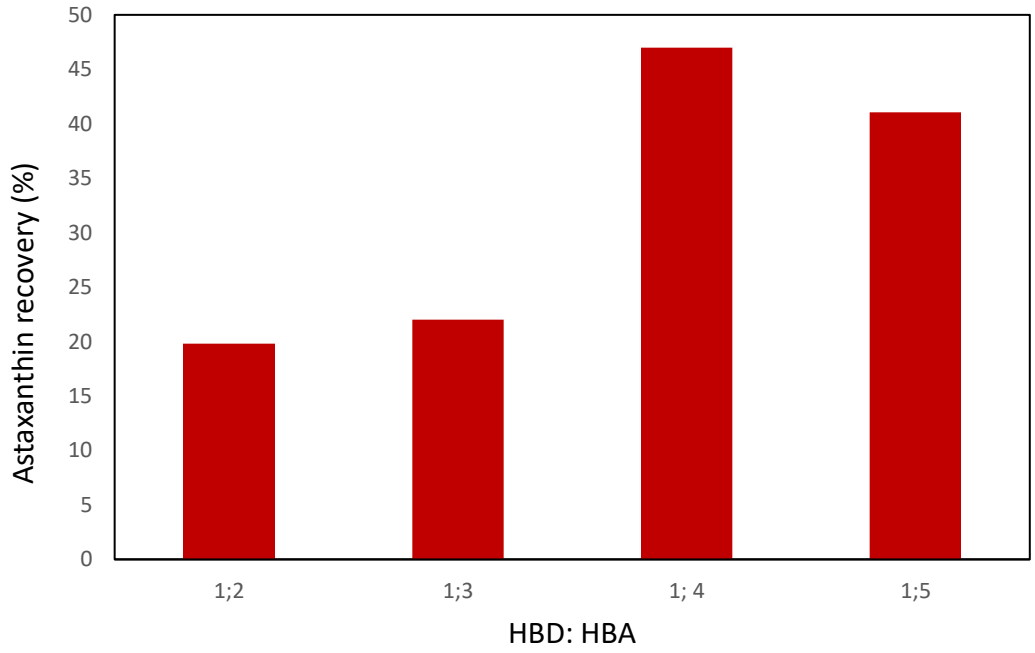

Figure 18: Astaxanthin recovery for different HBD: HBA molar ratios of ChCl-G. HBD: HBA ratio of 4 had a better astaxanthin recovery (%) compared to the other ratios.

It was found that a high HBD: HBA ratio leads to high astaxanthin recovery which is in agreement with the study done by H. Zhang et al. Figure 18 shows that the HBD: HBA ratio of 4 has the highest astaxanthin recovery among the ratios tested. This result could be due to viscosity and polarity change (Q. Zhang et al., 2012).

Table 10: Polarity of different molar ratios of ChCl-G (Q. Zhang et al., 2012)**.**

Solvent	HBA: HBD	E_T (30)/kcal mol^{-1}
ChCl-G	1: 3	57.96
ChCl-G	1: 2	58.28
ChCl-G	1: 1.5	58.21

Table 11: Viscosity of different molar ratios of ChCl-G.

Salt (HBA)	HBD	HBA: HBD	Viscosities (cP)	Reference
ChCl	Glycerol	1: 5	500 (25 °C)	(Abbott et al., 2011)
ChCl	Glycerol	1: 4	440 (25 °C)	(Abbott, Harris, et al., 2007)
ChCl	Glycerol	1: 3	400 (25 °C)	(Abbott, Harris, et al., 2007)

Table 10 and Table 11 show how polarity and viscosity of ChCl-G change as the amount of glycerol in the system is increased. The table indicates that the polarity of the system decreases as more glycerol is added, which is a favorable for astaxanthin extraction. The addition of more glycerol to the system increases the viscosity of DES which hinders the extraction efficiency. According to Figure 18, when the molar ratio of glycerol increases, the positive impact of polarity change is greater than the negative impact of viscosity change and increases astaxanthin recovery until the ratio reaches 4. Further increase in the molar ratio of glycerol results in a decrease in extraction efficiency because the negative effect of viscosity gets larger than the positive effect of polarity.

ChCl-G mixture was chosen for further research and optimization of experimental conditions. It also consists of glycerol which is more biodegradable and non-toxic compared to ethylene glycol.

4.3 Central composite design (CCD)

In the screening experiments detailed in the previous section, the factors tested showed a significant impact on astaxanthin recovery and also demonstrated trends with convex curve indicating the existence of an optimum point. In order to determine the optimum conditions for astaxanthin extraction, the central composite design was employed for optimization. This design also provides significant insight into the interactions and the effects of the parameters on the astaxanthin recovery. Temperature, molar ratio of HBD: HBA, volume ratio of DES: water, and time were selected to study their individual and interaction effects on extraction.

Table 12: Coded and uncoded experimental conditions used in CCD

	Coded levels				
Factors	-2	-1	0	1	2
Temperature (°C)	40 °C	50 °C	60 °C	70 °C	80 °C
HBD: HBA	0.5	2.5	4.5	6.5	8.5
DES: water	1	3	5	7	9
Time (h)	3	6	9	12	15

Among the models tested, the quadratic model showed a better fit than other models. The quadratic model allowed us to optimize and detect any curvature effect that factors might have.

$$\boldsymbol{Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i<j}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j} \qquad (2)$$

Experimental conditions tested in this study and the results of the runs using choline chloride-glycerol (ChCl-G) are summarized in Table 13. Astaxanthin recovery (%) was used as the response in the model which was calculated as the percentage of astaxanthin extracted (mg) to the total astaxanthin (mg) available in microalgae for the extraction.

$$\boldsymbol{Astaxanthin\ recovery} = \frac{\boldsymbol{Astaxanthin\ extracted\ using\ DES\ (mg)}}{\boldsymbol{Total\ astaxanthin\ available\ (mg)}} * \mathbf{100} \quad (3)$$

The total available astaxanthin was estimated to be the amount extracted from freeze-dried *H. pluvialis* cells disrupted using a mini-bead beater for 5 minutes followed by an extraction step using conventional organic solvents, using chloroform (2 ml) and dimethyl sulfoxide (4 ml) as solvents. The batch used for CCD experiments contained 1.3% astaxanthin on a dry weight basis.

Under the experimental conditions used, astaxanthin recovery was within the range of 15 to 68% (w/w).

Table 13: Experimental conditions and results of (CCD) for extraction.

#	Temperature (°C)	HBD/ HBA (mol/mol)	DES/Water (V/V)	Time (hr)	Astaxanthin recovery (%w/w)
1	70	2.5	3	6	29
2	50	6.5	3	6	41
3	70	2.5	7	6	26
4	50	6.5	7	6	15
5	70	6.5	7	6	22
6	50	2.5	3	12	47
7	70	2.5	3	12	32
8	50	6.5	3	12	42
9	70	6.5	3	12	35
10	50	2.5	7	12	43
11	70	2.5	7	12	64
12	50	6.5	7	12	53
13	70	6.5	7	12	68
14	40	4.5	5	9	60
15	80	4.5	5	9	47
16	60	8.5	5	9	39
17	60	4.5	1	9	19
18	60	4.5	9	9	29
19	60	4.5	5	3	30
20	60	4.5	5	15	59
21	60	4.5	5	9	54
22	60	4.5	5	9	55
23	60	4.5	5	9	53
24	60	4.5	5	9	52
25	60	4.5	5	9	57
26	60	4.5	5	9	52
27	60	0.5	5	9	50

In this model, "*T*" indicates temperature, "*M*" molar ratio of HBD to HBA, "*V*" the volume ratio of DES to water, and "*t*" is the time of extraction.

In order to simplify the model, insignificant terms were removed based on their p-value. When removing the terms, parameters such as lack of fit, adjusted-R^2, and R^2 were also taken into account. The two terms, the second order of temperature (T^2) and the interaction term between temperature and molar ratio of HBD: HBA (*T-M*), were also removed from the model due to their insignificance and poor contribution to the model.

The model was recalculated based on stepwise deletion of insignificant terms. Table 14 shows the analysis of variance (ANOVA) for the rearranged model when T^2 and *T-M* terms are not included.

Table 14: Analysis of Variance (ANOVA) for the modified Quadratic model

Variables	Sum of squares	Degree of freedom	F-value	p-value	
Model	5253	12	37.8	< 0.0001	Significant
T	131.7	1	11.4	0.0046	
M	115	1	9.9	0.0071	
V	50.3	1	4.3	0.0560	
t	1459	1	126	< 0.0001	
T-V	639.4	1	55.2	< 0.0001	
T-t	175.3	1	15.1	0.0016	
M-V	46.5	1	4	0.0649	
M-t	153.3	1	13.2	0.0027	
V-t	725.1	1	62.6	< 0.0001	
M^2	202.4	1	17.5	0.0009	
V^2	1714.2	1	148	< 0.0001	
t^2	196.7	1	17	0.0010	
Residual	162.1	14			
Lack of Fit	142.4	9	3.9	0.0699	Not significant
Pure Error	19.7	5			

The F-value for the model is 37.8, which shows that the model is significant and there is only 0.01% chance that an F-value this large could occur due to noise. As indicated in Table 14, the lack of fit for the model is not significant and it shows that the model fits the data. The fit summary of the model is shown in Table 15.

Table 15: Fit summary of the model

Std. Dev.	3.4	R^2	0.97
Mean	43	Adjusted R^2	0.9
C. V. %	8	Predicted R^2	0.8
		Signal/noise	22

The high R^2 and adjusted R^2 are good indications that the selected model can explain the variability of astaxanthin recovery. Signal to noise ratio for the model is 22, greater than 4, and implies that the model has adequate signal and it can be used to navigate the design space. The coefficient estimates of the model for single factor and interaction terms are presented in Table 16.

Table 16: Coefficient Estimates

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High
Intercept	53	1.2	50.6	55.7
T	-2.8	0.8	-4.6	-1
M	-2.6	0.8	-4.4	-0.8
V	1.6	0.8	-0.1	3.3
t	8.6	0.8	7	10.2
T-V	7.5	1	5.3	9.7
T-t	4.3	1.1	1.9	6.7
M-V	2	1	-0.1	4.2
M-t	4	1.1	1.7	6.4
V-t	7.9	1	5.7	10
M^2	-2.7	0.6	-4.1	-1.3
V^2	-7.9	0.6	-9.3	-6.5
t^2	-2.7	0.6	-4.1	-1.3

Table 16 shows the significant effects of the components and process parameters on astaxanthin recovery. There is a strong interaction between factors except for the temperature and HBD: HBA ratio, and therefore true analysis of the effects cannot be done based on a single factor. The negative contributions of second-order terms of V^2 and T^2 compared to their positive effects indicates that the surface is convex. In other words, the positive effect of time and volume ratio on astaxanthin recovery decreases as their value increases.

As an evaluation of the accuracy of the adopted model, two plots, predicted versus actual data and residuals versus predicted, are shown in Figure 19.

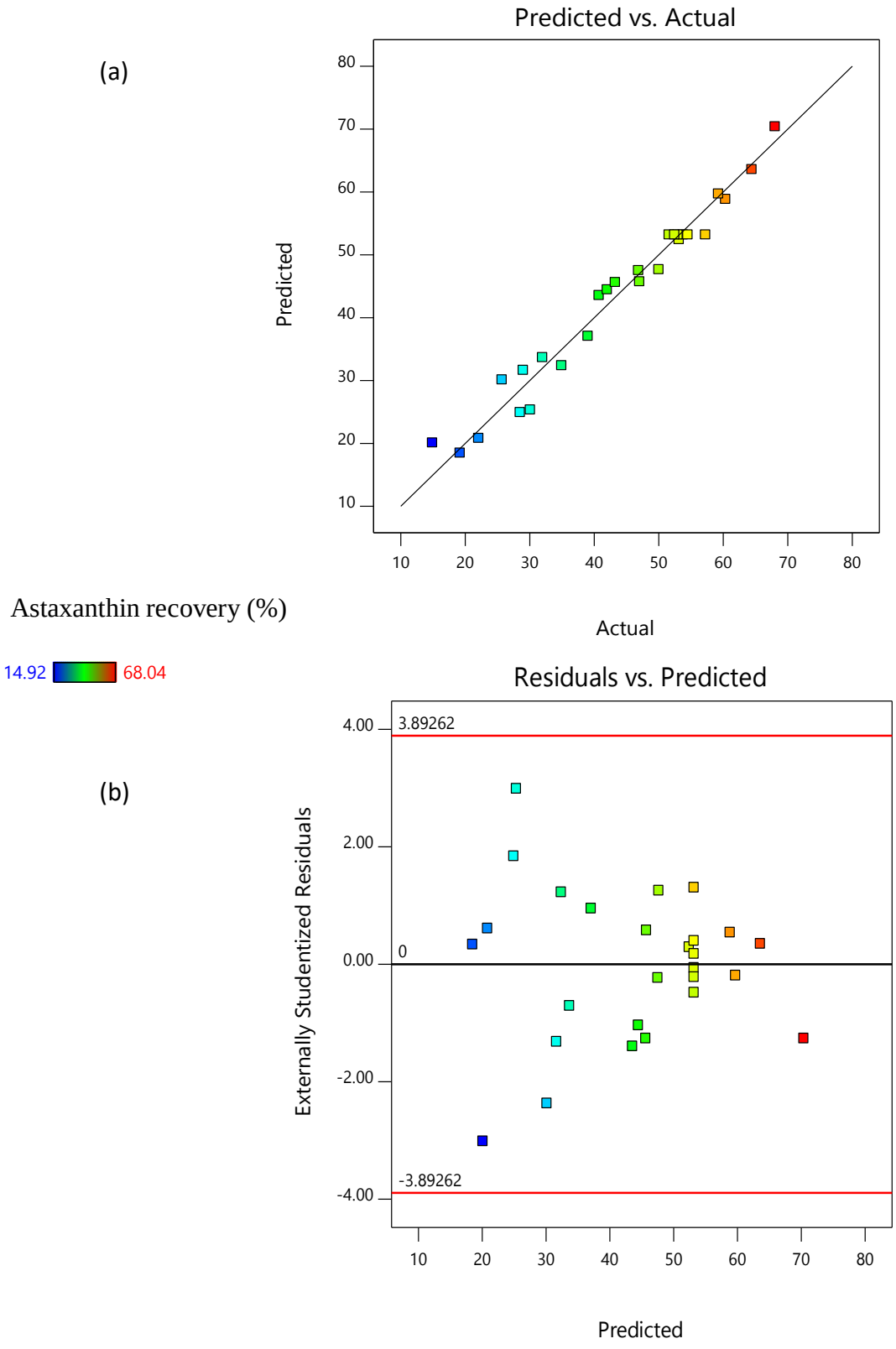

Figure 19: (a) predicted vs. actual data; (b) residuals vs. predicted values.

Figure 19 (a) shows that the points, which represent actual values, are very close to the fitted line, which indicates that the predicted data was in a good agreement with and strongly correlated to the observed results. The residuals in Figure 19 (b) are within the limits and there is not a clear pattern in the plot, which implies the accuracy of the selected model.

4.3.1 Single factor effects in the model

The effect of factors on astaxanthin recovery regardless of their interactions with other factors have been investigated in the design space.

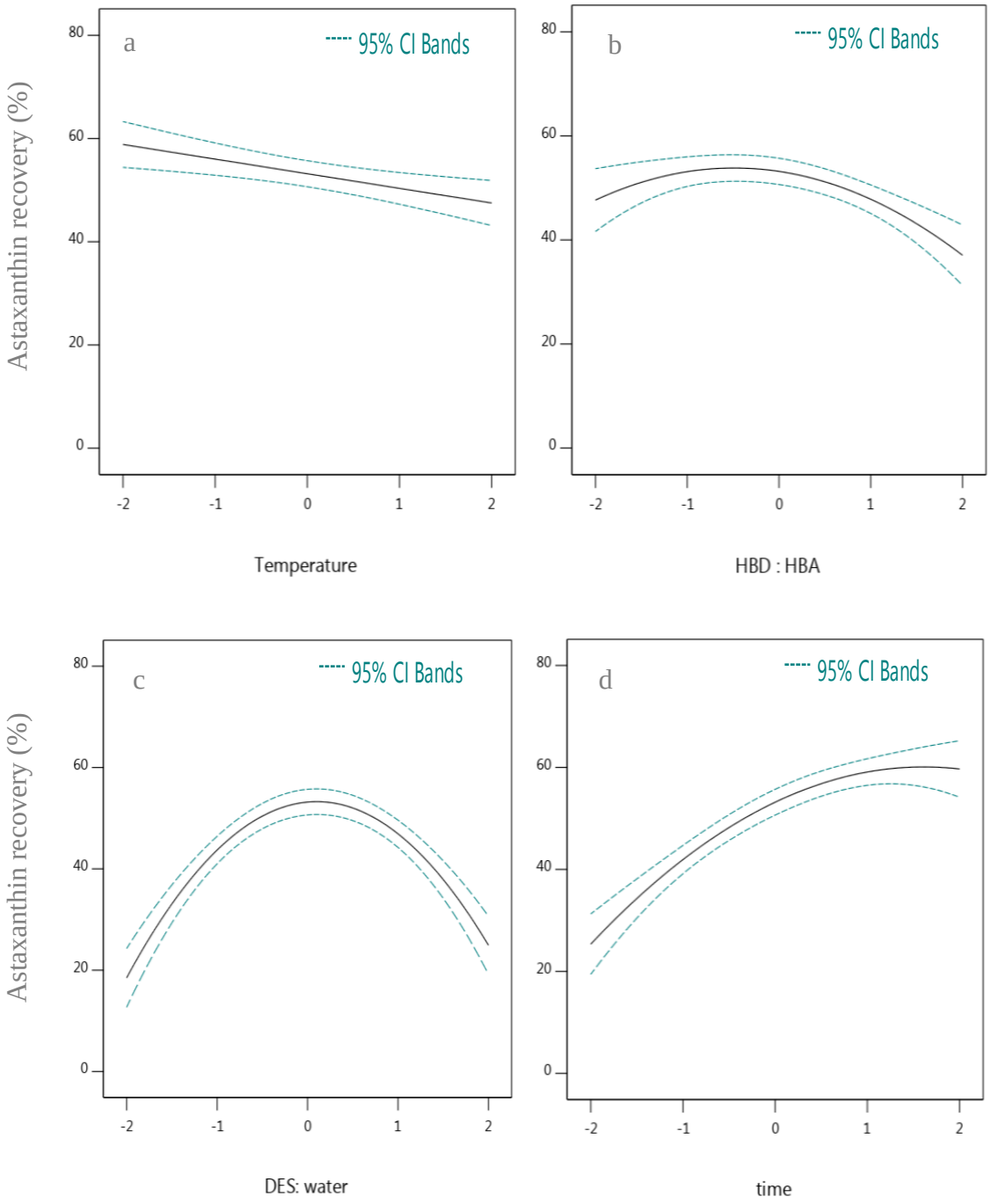

Figure 20: Single factor effects on astaxanthin recovery. (a) The effect of temperature on astaxanthin recovery. (b) The effect of HBD: HBA molar ratio on the extraction. (c) The effect of the volume ratio of DES: water on the extraction (d) The effect of time on astaxanthin extraction. The factors other than the one being investigated were set to 0, center point. Temperature increase had a negative

impact. HBD: HBA and DES: water had an optimum around the center point. Increasing the duration of extraction improved astaxanthin recovery.

Given the model and coefficients, all factors have interaction with each other. Figure 20(a) demonstrates that increasing temperature has a negative effect on the astaxanthin recovery. This behavior, in the design range, 50- 70 °C, is in agreement with the results that Reyes et al. obtained when investigating the effect of temperature on astaxanthin recovery using CO_2-expanded ethanol. The temperature range that they studied was 30 to 60 °C and astaxanthin recovery shows a negative correlation with temperature for the temperatures more than 45 °C. This could be because of the interactions of the factors and astaxanthin degradation at high temperatures. There are some studies that suggest that increasing the temperature makes the experimental conditions favorable for high astaxanthin recovery (Machmudah, Shotipruk, Goto, Sasaki, & Hirose, 2006). A similar behavior is observed in Figure 20(b) for the impact of HBD: HBA ratio on astaxanthin recovery to the one showed in Figure 18. It is clear that the optimum point is around the center point, which has an HBD: HBA ratio of 4.5. When HBD: HBA changes, the viscosity and polarity of the solution are also altered. Higher astaxanthin recovery around the center point indicates that this point represents better conditions for the extraction process. Figure 20(c) shows the effect of having more volume of DES compared to water in the system. Adding more DES to the system, until the volume ratio reaches to 5, enhances astaxanthin recovery. This enhancement can be attributed to the increase in the hydrogen bonds of the system, which improves astaxanthin recovery. Conversely, when the system contains DES at a greater than optimal ratio, around center point, the viscosity of the solution increases and results in a reduction in astaxanthin recovery. These results are also compatible with the results found in the literature (H. Zhang et al., 2014). The last section of Figure 20 demonstrates that astaxanthin recovery changes in relation to time. As it was expected, increasing the time of extraction led to greater astaxanthin recovery. More contact time increased mass transfer from microalgae to the solution, which in turn enhanced astaxanthin recovery. However, this increase stopped after the solution became saturated.

4.3.2 Central composite design response surface

One of the advantages of using DoE is the ability to observe how different factors interact with each other. As discussed, time and astaxanthin recovery were positively correlated. In order to see the interaction among other factors of the system, contour plot of HBD: HBA versus temperature is drawn.

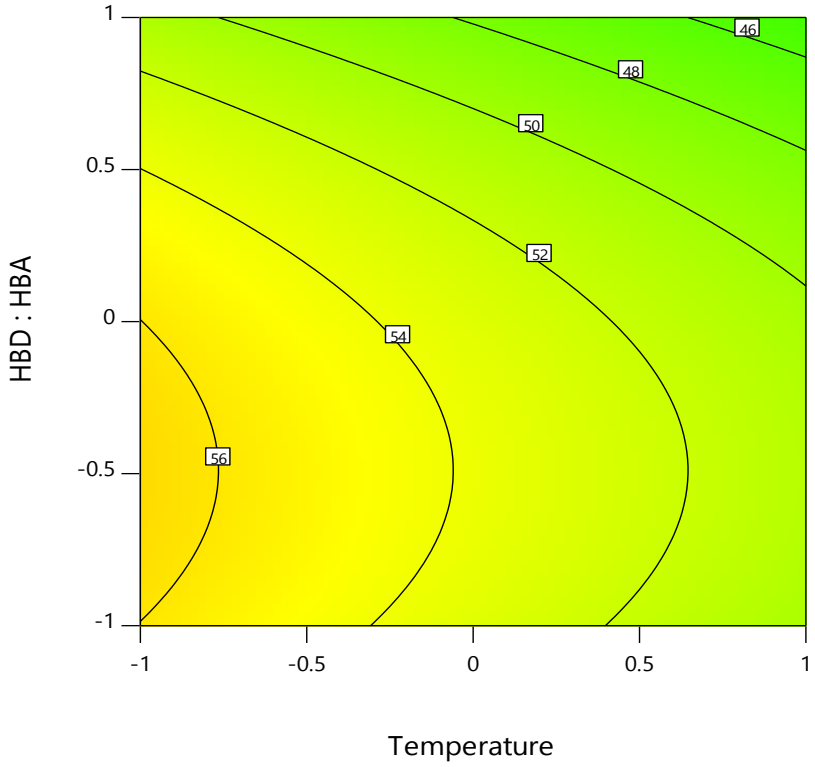

Figure 21: Contour plot of HBD: HBA ratio vs temperature when other factors are at the center point.

Considering just the factors, *M* and *T*, and when the other two factors are set at their center points, the optimum point falls below the center point. This could be due to physicochemical properties of the solvent used. When the portion of water in the system is increased, the contour plot shown in Figure 21 changes to the plot presented below:

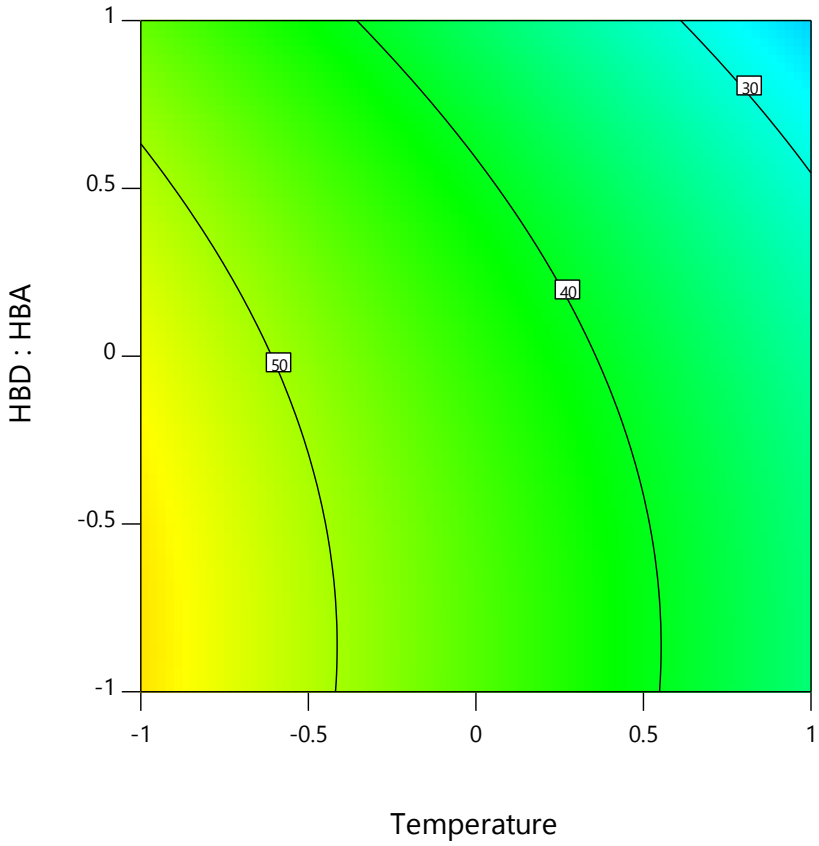

Figure 22: Contour plot of HBD: HBA ratio vs temperature when 25%(v) of the solution is water.

As Figure 22 shows, astaxanthin recovery was decreased by increasing the amount of water in the system to 25% of the total volume. At this point, the number of hydrogen bonds has decreased as a result of having water more than it is needed. And the optimum conditions have shifted to a higher HBD: HBA ratio and temperature. To compensate for the reduction in the hydrogen bonds caused by having too much water, the system needs a higher portion of the HBD component to maintain a high astaxanthin recovery. To investigate how astaxanthin recovery changes when the amount of water present in the system drops to 12.5%, the contour plot for *M* and *T* has been drawn when *V*=1.

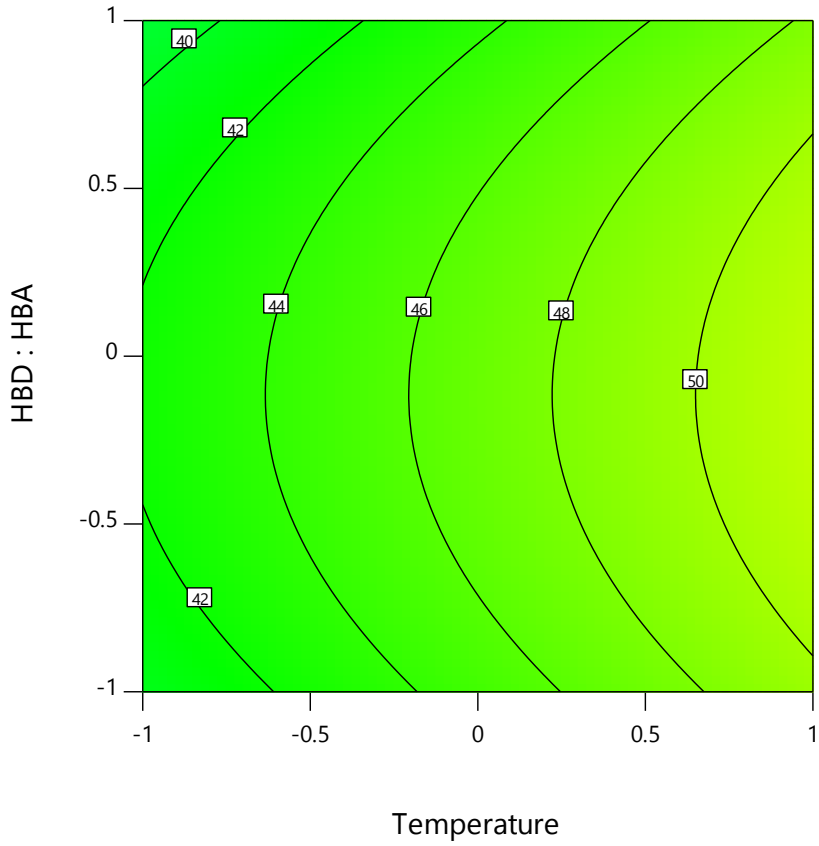

Figure 23: Contour plot of HBD: HBA ratio vs temperature when the amount of water is minimum.

The optimum point shifts to a higher temperature when the solution consists of 87.5% DES and 12.5% water, as can be seen in Figure 23. DES used, ChCl-G, has a higher viscosity than water and an increase in DES portion of the solution leads to an increase in the system viscosity which hinders mass transfer and astaxanthin recovery. In order to keep the viscosity of the system low, higher temperature is needed. Optimum HBD: HBA ratio is almost constant and around its center point.

4.3.3 Central composite design optimization

The overall goal of using CCD is to optimize the experimental conditions over design space. To confirm the model, experiments were run in the design space an also in the area close to the star points. It was found that the model has a good predictability for design space. However, it did not qualify as a satisfactory model for predicting outside the

design space. Numerical optimization of astaxanthin recovery was performed using Design Expert 11 with the following conditions:

- All the factors stay in the range, -1 to +1
- All were set to the same importance
- The response, astaxanthin recovery, were set to be maximized and the limits were set to be within the 15 to 100%.

These conditions resulted in very few solutions. The optimal astaxanthin recovery predicted to be achieved under these restrictions within the design space is about 70%. The operating conditions for the optimum point are 70 °C, an HBD: HBA ratio around 5.5, water content around 12% and 12 hours of extraction.

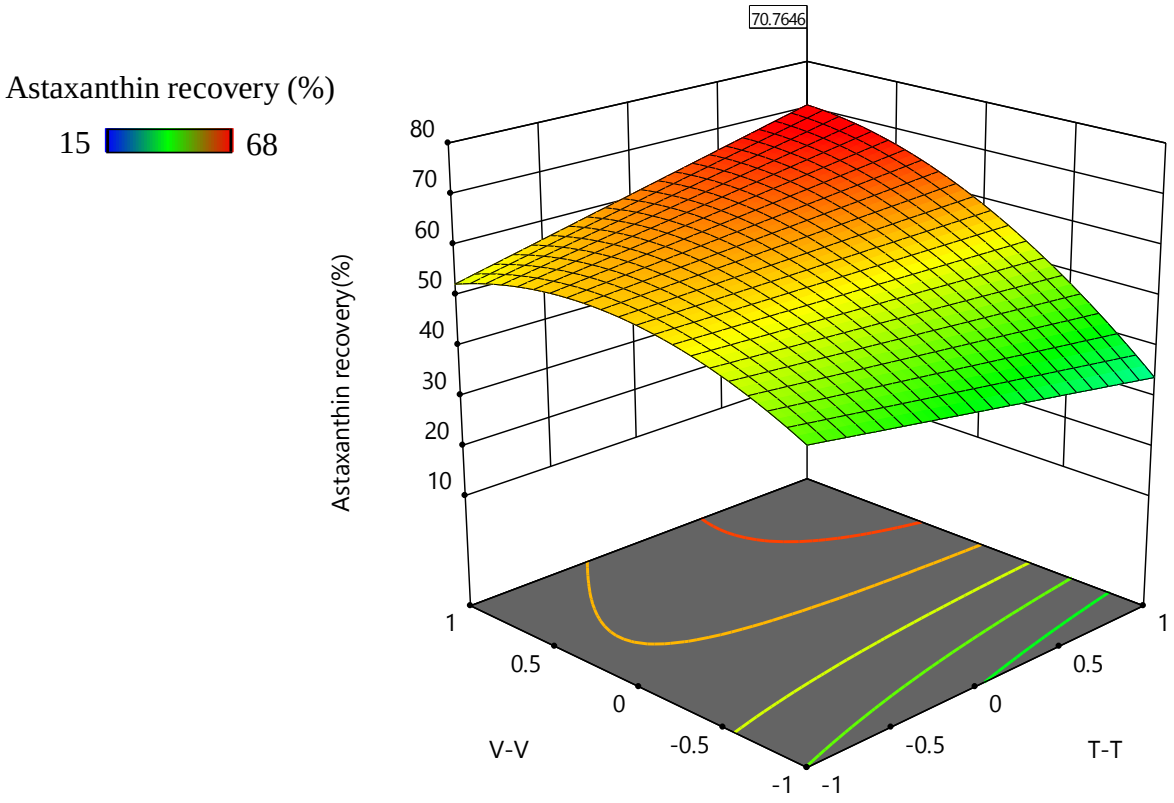

Figure 24: Surface plot of the optimum point.

Figure 24 shows a 3D response surface plot for the highest astaxanthin recovery that the model predicts within the design limits considering the interactions between factors that were considered in the model.

4.4 Recovery of extracted astaxanthin

In this work, the samples after extraction with DES, containing 20 mg dry *H. pluvialis*, were diluted with dimethyl sulfoxide (DMSO) for the analysis which will not be a required step when large amounts of *H. pluvialis* used for the extraction. Due to the solubility of astaxanthin in DES-water systems, astaxanthin extracted astaxanthin rises to the surface of the solution after it is centrifuged. When the amount of microalgae used for extraction is large which will lead to large amounts of astaxanthin, and the astaxanthin can be collected from the top surface.

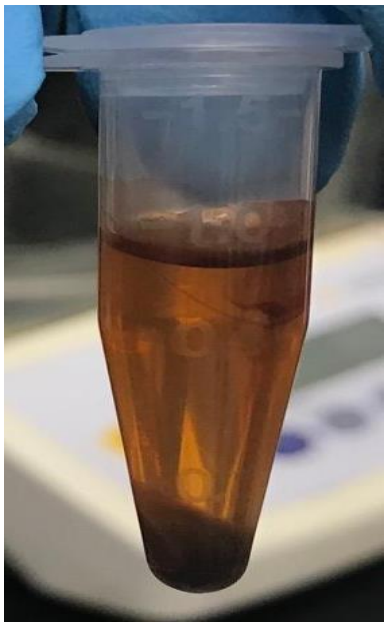

Figure 25: Centrifuged samples after extraction.

As it is shown in Figure 25, there is a top layer that contains large amounts of astaxanthin that could be recovered easily when the extraction is on a large scale.

Chapter 5

5 Conclusions and future work

5.1 Conclusions

In conclusion, DESs are capable of disrupting the cell wall of *H. pluvialis* and the use of DES allows the direct extraction of astaxanthin. Furthermore, extraction by DES-water mixture allows easy and simple recovery of astaxanthin due to the solubility of astaxanthin in the system. The optimal conditions for operating parameters were determined: 70 °C, an HBD: HBA ratio around 5.5, water content around 12% and 12 hours of extraction. The effects of adding co-solvent to the system on astaxanthin recovery was also demonstrated.

Overall this work contributed to our greater understanding of the fields that deep eutectic solvents could be used.

5.2 Future work and recommendation

In this study, the central composite design used had poor predictability of design parameters outside the design space. Therefore, future work could be done outside the design space to gain a better insight into how astaxanthin recovery is affected by factors outside the design limits.

This work demonstrated that DES could extract astaxanthin from dry *H. pluvialis* and the results obtained open new avenues for astaxanthin extraction. Future work could be done to find out how DES would perform when used for wet microalgae extraction.

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Appendix

Chemical Society reviews

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Curriculum Vitae

EDUCATION

Master of Engineering Science in Chemical Engineering **Sep. 2016-Dec.2018**

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Bachelor of Science in Chemical Engineering **Sep. 2011-Sep.2015**

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Amirkabir University of Technology, Tehran, Iran

ACADEMIC PROJECTS

Master Thesis "Extraction of astaxanthin from microalgae by deep eutectic solvents" **Sep. 2016-Dec.2018**

- Investigating the optimal operation condition for the extraction process
- Determining the highest achievable efficiency with this treatment
- Studying the feasibility of this method in industry scale

Bachelor Thesis "Investigation of fine particles and pollution sources by Bayesian Inverse method" **Sep. 2014-Sep.2015**

- Used Weather Research and Forecasting (WRF) model to simulate the observed data
- Determined the amount of fine particle dispersed by each region
- Tracked the sources of pollution by Bayesian Inverse method

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Graduate Teaching Assistant **Jan. 2018-Apr.2018**
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PUBLICATION

L. Roshanfekr, M. Irani, R. Barzegar. “Adsorptive removal of acetaminophen and diclofenac using NaX nanozeolites synthesized by microwave method”, published in Korean Journal of Chemical Engineering, August 2015, Volume 32, Issue 8, pp 1606-1612.